THE UNIVERSITY *of* EDINBURGH

The exosomal proteome as a source of biomarkers for human disease.

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Declaration

I hereby declare that all work described in this thesis, except for some aspects of the procedures highlighted in the acknowledgements, was performed entirely by myself. With the exception of the analysis of cJun in the urinary exosomes of patients with sepsis (Figure 3.7) the work contains no material that has been accepted for the award of any other degree or diploma in any university or tertiary institution. To the best of my knowledge no material published or written by any other person, except where indicated in the text, is included in this thesis.

Jonathan Mark Street

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Abstract

Exosomes are small lipid membrane bound vesicles formed as part of the endosomal pathway and released into the extracellular space following fusion of late endosomes with the plasma membrane. Exosomes have been shown to have a variety of biological roles and may represent a novel source of disease biomarkers.

The objectives of this project were to develop a panel of techniques for identifying exosomes in human urine then establish an in vitro model to determine whether exosomes change with cellular activation. We then used the techniques developed with human urine to determine whether human cerebrospinal fluid (CSF) contains exosomes and applied a mass spectrometry based approach to characterise the exosomal proteome.

We used western blot for three exosomal markers (tsg101, CD24 and flotillin-1), isopycnic centrifugation on a sucrose density gradient and direct visualisation using transmission electron microscopy (TEM) to verify the presence of exosomes. Using GeLC-MS/MS, 88 proteins were identified in the urinary exosomes. Several of these proteins could be linked to diseases and specific sections of the nephron.

A murine cortical collecting duct cell line was used to model exosome release into the urine. Firstly, exosome release was verified using the approach developed in the urine. Stimulation of the cells with desmopressin caused an increase in the presence of aquaporin 2 in the exosomes. This increase

reflected a similar change in the cells and occurred over a similar time course. This supports the hypothesis that the exosomes reflect the state of the kidney cells. In contrast, stimulation with cisplatin did not alter the presence of Fetuin-A, a proposed biomarker of cisplatin-induced acute kidney injury, in exosomes and this was consistent with no change in Fetuin-A expression in the cells.

The released exosomes may act as mediators of communication to other cells. Following incubation of mCCD cells with AQP2 containing exosomes AQP2 in the cell lysate was increased indicating interaction between the cells and exosomes and potentially internalisation.

Exosomes have been shown to be released by neuronal cells *in vitro*. We identified exosomes in the CSF of humans using western blot for known exosomal markers, density determination and direct visualisation with TEM and Immuno-TEM using an antibody specific for the exosomal marker flotillin-1. Label-free quantitative mass spectrometry was used to compare multiple CSF samples. On a whole protein analysis 86% of the proteins identified varied by less than 2-fold in comparison to the average across samples. On a tryptic peptide analysis 75% of the peptides identified varied by less than 2-fold in comparison with the average across samples.

We have demonstrated exosomes are present in urine, CSF and mCCD cell conditioned media. In the mCCD cell derived exosomes we have demonstrated that following stimulation the proteome of the exosomes changes and that this change reflects the change seen in the cells. For the urinary and CSF exosomes we have characterised their proteomes using

GeLC-MS/MS. These findings are consistent with the hypothesis that exosomes are a rich source of information, including biomarkers, on their cells of origin.

Presentation of Work

Manuscripts in Preparation

Aquaporin 2 is released from and transferred between mCCD cells in exosomes.

Identification and proteomic analysis of exosomes in human cerebrospinal fluid.

Published Articles

Street JM, Dear JW. The application of mass-spectrometry-based protein biomarker discovery to theragnostics. British Journal of Clinical Pharmacology 2010; 69: 367-78.

Oral Presentation

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Street J, Barran P, McAllister F, Webb D, Dear J. Proteomic investigation of human urinary microparticles. British Pharmacological Society Winter Meeting 2008

Poster Presentation

Street JM, Bailey MA, Webb DJ, Dear, JW. Development of an in vitro Model to Investigate Changes in Kidney-derived Exosomes with Disease. ASN Renal Week November 2010

Street JM, Chalmers RTA, Walsh TS, Webb DJ, Dear JW. Human cerebrospinal fluid contains exosomes that represent a novel reservoir for therapeutic biomarker discovery. WorldPharma July 2010

Street JM, Bailey M, Frame F, Walsh TS, Webb DJ, Dear JW. Development of an in vitro model to investigate changes in kidney-derived exosomes with disease. WorldPharma July 2010

Street JM, Chalmers RTA, Walsh TS, Webb DJ, Dear JW. Human cerebrospinal fluid contains exosomes that represent a novel reservoir for therapeutic biomarker discovery. British Pharmacological Society Winter Meeting 2009

Street JM, McAllister F, Bailey M, Walsh TS, Webb DJ, Barran P, Dear JW. Urinary exosomes are a novel reservoir for biomarker discovery. British Pharmacological Society Winter Meeting 2009

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Street JM, McAllister F, Bailey M, Walsh TS, Webb DJ, Barran P, Dear JW. Urinary exosomes are a source for biomarker discovery in acute kidney injury. Congress of the European Association for Clinical Pharmacology and Therapeutics 2009

Street J, McAllister F, Webb DJ, Barran P, Dear J. Proteomic investigation of human urinary exosomes as a source for biomarker discovery. Scottish Society for Experimental Medicine, Spring 2008 Conference.

Stevenson SF, Street JM, Webb DJ, Dear JW. The detection and qualification of RNA within human urinary exosomes. Scottish Society for Experimental Medicine, Spring 2008 Conference.

Abbreviations

AKI	Acute Kidney Injury
AQP2	Aquaporin 2
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CE-MS	Capillary Electrophoresis - Mass Spectrometry
CHMP	Charged MVB Protein subunit
CID	Collision Induced Dissociation
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
dDAVP	Desmopressin
DIGE	Difference In Gel Electrophoresis
DMEM	Dulbecco's Modified Eagle's Media
DPBS	Dulbecco's PBS
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
ESCRT	Endosomal Sorting Complex Required for Transport
ESI	Electrospray Ionoisation
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FLUOS	Fluorescein
FTICR	Fourier Transform Ion Cyclotron Resonance
GeLC-MS/MS	LC-MS/MS with prefractionation by SDS-PAGE

HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPLC	High Performance Liquid Chromatography
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
ICAT	Isotope-Coded Affinity Tags
IgG	Immunoglobulin
IT	Ion trap
iTRAQ	Isobaric Tag for Relative and Absolute Quantitation
ITS	Insulin Transferrin Selenium
KIM-1	Kidney Injury Molecule 1
LBPA	Lyso-bisphosphatidic acid
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography couple tandem mass spectrometry
LDS	Lithium Dodecyl Sulphate
MALDI	Matrix Enhanced Laser Desoprtion Ionoisation
MARS	Multiple Affinity Removal System
mCCD	Murine Cortical Collecting Duct
MDP	Modular toolkit for Data Processing
MES	2-(N-morpholino)ethanesulfonic acid
MS	Mass spectrometry
NAG	N-acetyl-β-glucosaminidase
NGAL	Neutrophil Gelatinase-associated Lipocalin
NIH	Nationa Institutes of Health
OMIM	Online Mendelian Inheritance in Man

PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PHP	Putative Heart Protein
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene Fluoride
Q	Quadrupole
RIPA	Radioimmunoprecipitation assay
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Denaturing Polyacrylamide Gel Electrophoresis
SELDI	Surface Enhanced Laser Desoprtion Ionisation
SEM	Standard Error about the Mean
SILAC	Stable Isotope Labelling by Amino acids in Culture
STAM	Signal Transducing Adaptor Molecule
TBST	TRIS Buffered Saline - Tween 20
TEM	Transmission Electron Microscopy
TOF	Time-of-flight
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
TSG	Tumor Susceptibility Gene

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1. Introduction

1.1. Exosomes

Cells release various types of membrane vesicles under physiological and pathological conditions (Figure 1.1). Although these vesicles vary widely in origin they share several common traits. They are all approximately spherical, contain soluble proteins in their lumen and are bounded by a lipid bilayer similar to other membranes in the cell. This lipid bilayer displays the luminal surface of the membrane from which the vesicle formed. For example, a vesicle formed from the plasma membrane will outwardly display the extracellular surface of the plasma membrane. This is in contrast to intracellular vesicles which enclose their luminal surface [1]. The released membrane vesicles vary in site of origin, size, density, and protein and lipid content (Table 1.1). This heterogeneity is not always clear in published articles where the terminology for different particles may be confused [2]. The term microparticle is often used to describe membrane vesicles which have not been thoroughly characterised or samples which may be a mixture of vesicles of different types, such as might be obtained from complex biofluids such as plasma.

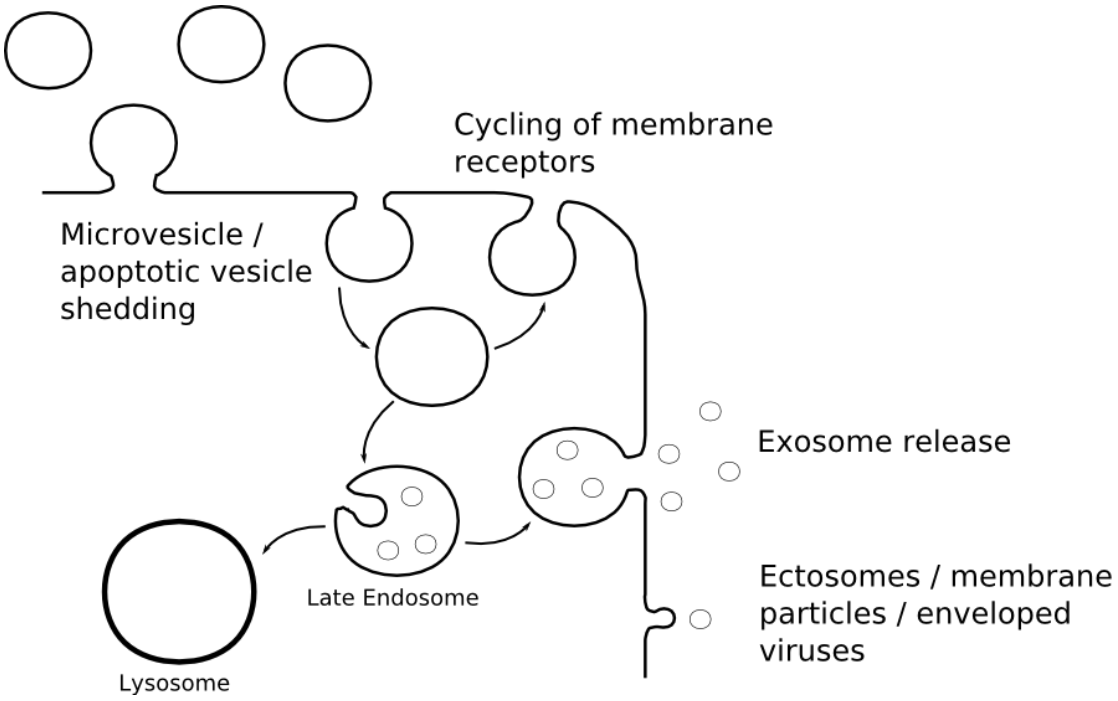


Figure 1.1 The cell releases a variety of different membrane vesicles.
The formation and release of vesicles occurs at two principal sites; the plasma membrane and intracellularly. The largest spectrum of vesicles is released from the plasma membrane ranging from the larger microvesicles and apoptotic vesicles to the smaller ectosomes and membrane particles. This method of release can also be utilised by enveloped viruses to mediate their release from cells. In contrast exosomes form intracellularly within late endosomes and only exit the cell following fusion of the late endosome with the plasma membrane.

Name	Size (nm)	Density (g/ml)	Appearance in TEM	Lipid composition	Main protein markers	Intracellular origin	Main reference
Exosomes	20-100	1.10-1.15	Cup shaped	Contain lipid rafts, enriched in cholesterol, sphingomyelin and ceramide	TSG101, Alix, flotillin 1, tetraspanins	Late Endosomes	[3]
Exosome-like vesicles	20-50	1.10	Irregular	Do not contain lipid rafts	TNFRI	?	[4]
Microvesicles	100-1000	?	Electron-dense and irregular	Expose phosphatidylserine	Integrins, selectins, CD40 ligand	Plasma membrane	[5]
Ectosomes	50-200	?	Round	Expose phosphatidylserine	CR1, L-selectin and proteases	Plasma membrane	[6]
Membrane particles	50-80	1.03-1.07	Round	Enriched in cholesterol and diacylglycerol	CD133	Plasma membrane	[7]
Apoptotic vesicles	50-500	1.18-1.28	Heterogeneous	?	Histones	?	[8]

Table 1.1 Characteristics of the different types of membrane derived vesicles.

1.1.1. Exosomal Biogenesis

Exosomes are formed as part of the endosomal pathway within eukaryotic cells. Invagination of the plasma membrane forms early endosomes which mature into late endosomes. During the maturation process the limiting membrane of the early endosomes undergoes invagination forming small vesicles in the lumen of the endosome. In the classical endosomal pathway the late endosomes fuse with the lysosomes and the contained vesicles and proteins are degraded. However, in 1987, it was demonstrated that the late endosomes can also fuse with the plasma membrane which releases the contents of the late endosome into the extracellular space [3]. The released vesicles are termed exosomes.

The invagination of the limiting membrane of the endosome is a multi-step process orchestrated by the Endosomal Sorting Complex Required for Transport (ESCRT) complexes (Figure 1.2). The protein hepatocyte growth factor-regulated tyrosine kinase Substrate (HRS) is targeted to endosomes via a FYVE domain (a four amino acid sequence of phenylalanine, tyrosine, valine and glutamic acid), which binds phosphatidylinositol 3-phosphate. Once bound, HRS which together with Signal Transducing Adaptor Molecule (STAM)-1 and STAM2 are often termed ESCRT-0, interacts with Tumor Susceptibility Gene (TSG)-101 which is a constituent of the ESCRT-I complex. The ESCRT-I complex then recruits ESCRT-II which causes the assembly of the ESCRT-III complex. From small structurally related monomers the large polymeric ESCRT-III complex assembles. The final step which forms the intralumenal vesicles is poorly understood, but the prevalent opinion is that it involves the disintegration of the ESCRT-III complex by the action of the Charged MVB Protein subunit (CHMP)-4[9].

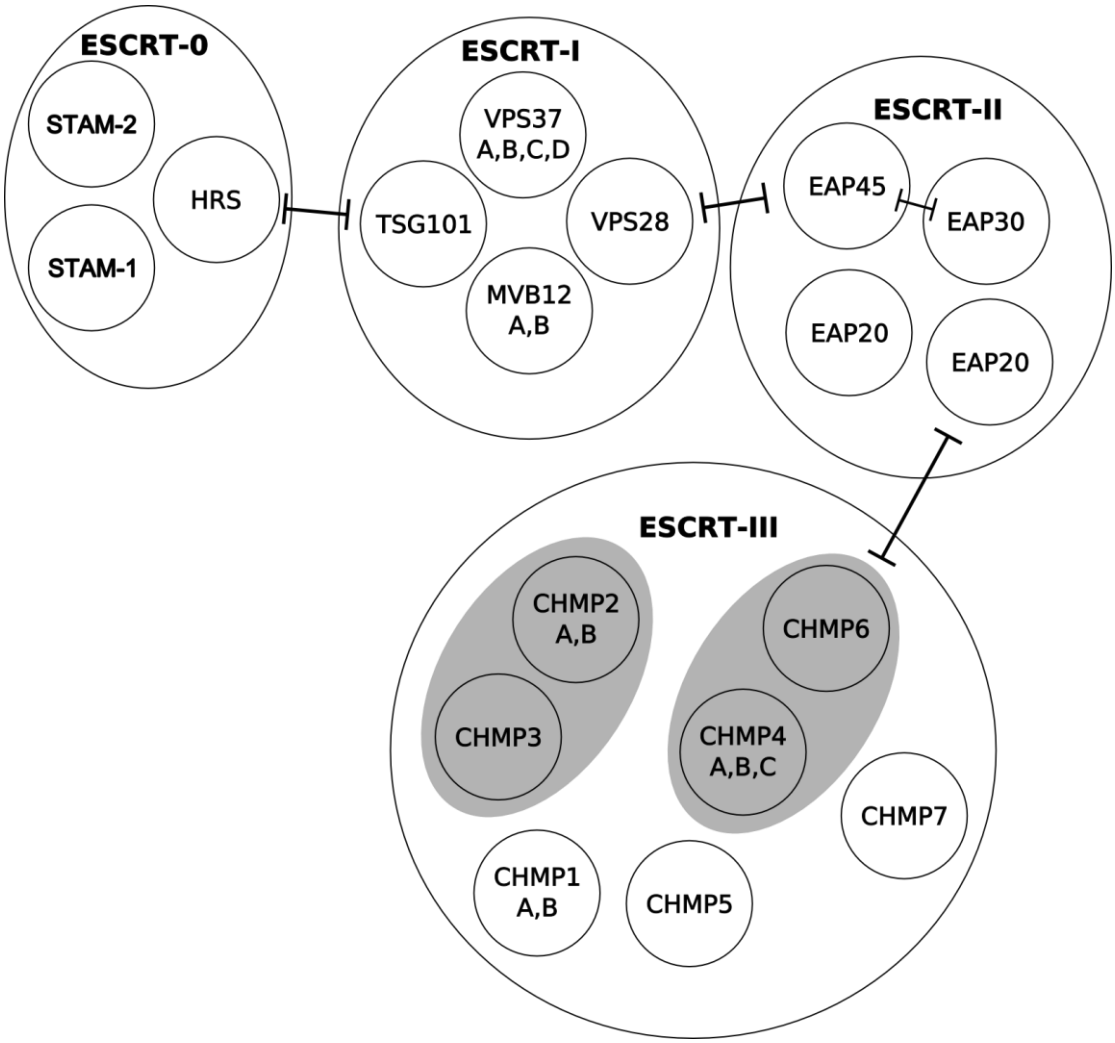


Figure 1.2 Schematic representation of the ESCRT complexes.
ESCRT-I and ESCRT-II exist as stable tetrameric complexes and are recruited in turn to the endosomal membrane following the interaction of the ESCRT-0 complex with phosphatidylinositol 3-phosphate in the membrane. Finally the components of the ESCRT-III complex, which exists as soluble monomers, assemble on the membrane and mediate membrane scission.

In addition to forming the intralumenal vesicles, the ESCRT complexes also mediate the inclusion of monoubiquitinated proteins into the intralumenal vesicles of late endosomes. The complexes ESCRT-0, -I and -II all contain ubiquitin binding domains which act to mediate the inclusion of monoubiquitinated cargo proteins into the endosomal pathway [9]. The STAM proteins present in the ESCRT-0 complex also recruit ubiquitin ligases and deubiquitinating enzymes which it is hypothesised release the ubiquitin from cargo proteins before they are encapsulated in the intralumenal vesicles [9]. Little is known about this process and, although the presence of deubiquitinating enzymes suggests ubiquitin is removed, many proteins in released exosomes are still associated with ubiquitin [10]. No ESCRT independent pathway for the inclusion of monoubiquitinated proteins in to intralumenal vesicles has been identified but monoubiquitination is not the only mechanism by which proteins are included in the intralumenal vesicles[11, 12]. Exosomes contain tetraspanins [13] and GPI-linked proteins which are known to be associated with lipid rafts [14]. Cholesterol and sphingolipids are enriched in both lipid rafts and exosomes suggesting that formation of intralumenal vesicles is enhanced at lipid rafts [1, 11]. In addition to their role in protein sorting into the intralumenal vesicles lipids may also be able to mediate the inwards invagination of the endosomal membrane which is required for vesicle formation. Lyso-*bis*phosphatidic acid (LBPA) has an unusual inverted cone shape which may favour inward invagination [15, 16]. Although present in mammalian cells LBPA is absent in yeast providing one possible explanation why ESCRT complexes are essential in yeast but not in mammalian cells for exosome formation [17].

1.1.2. Biological Activity

Since first being observed, evidence has accumulated that exosomes are more than inert particles to be expelled from the body and have a variety of biological roles mediated by their proteome (Table 1.2). In addition to release during reticulocyte maturation exosomes are also involved in morphogen signalling and a variety of immunological activities [18-20]. Argosomes, which appear identical to exosomes, have been described [21, 22] as a mechanism for the dispersal of morphogens, specifically Wingless in *Drosophila*, and the creation of the morphogen gradient. Exosomes have both immuno-stimulatory and immuno-inhibitory activities depending on the context. B cells which have been transformed with Epstein-Barr virus release exosomes which can activate CD4+ T cells. This activation is antigen specific [23]. Dendritic cells which have been pulsed with peptides from tumours release exosomes which support tumour rejection. Again, this activity is antigen specific [24, 25]. Exosomes released from melanoma [26], prostate [27] and mammary [28] tumour cells have been shown to mediate lymphocyte apoptosis and may be involved in tumour immune evasion. Intestinal epithelial cells release exosomes which may mediate antigen presentation in the mucosal and systemic immune system and be involved in tolerance [29]. During pregnancy exosomes of placental origin are present in the maternal circulation and may be involved in the deletion of reactive lymphocytes [30, 31]. Exosomes may also be involved in the progression of neurological disease. β-amyloid peptide which is involved in the pathogenesis of Alzheimer's disease is released from cells in association with exosomes [32]. Prion infectivity is released from neuroblastoma cells in association with exosomes *in vitro* [33-35] and *in vivo* is present in exosomes purified from the cerebrospinal fluid of sheep [36].

Cell of release	Mechanism	Target cell	Action	Reference
Reticulocyte	Exosome carries transferring receptor	-	Loss of transferring receptor during maturation	[3]
Ere reported Drosophila wing imaginal disc epithelium	Exosome carries Wingless	Developing tissues	Establishes morphogen gradient	[21, 22]
B cells	Antigen presentation	T cells	T cell activation	[23]
Dendritic cells	Antigen presentation	Tumour cells	Support tumour rejection	[24, 25]
Tumour cells	Exosome carries FasL	Lymphocytes	Lymphocyte apoptosis and tumour immune evasion	[26-28]
Intestinal epithelial cells	Antigen presentation	CD4+ T cells	Tolerance	[29]
Placental cells	Exosome carries FasL	T cells	Deletion of reactive lymphocytes	[30, 31]
Neuroblastoma cells	Exosome carries β-Amyloid	Neuroblastoma cells	Plaque formation in Alzheimer's disease	[32]
Neuroblastoma cells	Exosome caries the prion protein	Neuroblastoma cells	Progression of TSEs	[33-35]

Table 1.2 Summary of the protein mediated biological roles for exosomes.

1.1.3. Exosome Purification

Since first being observed during reticulocyte maturation it has been found that exosomes are released from a wide variety of cells for example, T and B [13, 23] lymphocytes, dendritic cells [37], mast cells [38], intestinal epithelial cells [29], platelets and neuroblastoma cells[35]. Although the number released varies, it appears that exosomes are released by all cell types *in vitro*. *In vivo* exosomes can be isolated from plasma [39], urine [10, 40], mouse and sheep cerebrospinal fluid [36, 41], human ascites fluid [42, 43], human and mouse amniotic fluid [40], human saliva [44] and mouse bronchoalveolar fluid [45].

In comparison to soluble proteins exosomes are present at a relatively low concentration in biofluids. For their study it is necessary to concentrate them preferentially compared with the soluble proteome. In the literature two methods have been used to achieve this; ultracentrifugation and molecular filtration [46]. Ultracentrifugation is by far the most common technique used but the necessary equipment may not be available in all laboratories and the process is time consuming. Molecular filtration has been used for the purification of exosomes only more recently. This approach holds promise as the necessary equipment is more commonly available and the process is relatively quick. Differences in the purified sample in comparison to ultracentrifugation have not yet been studied. Currently it is unclear how the samples prepared by ultracentrifugation and molecular filtration may differ, although some evidence is now emerging [47]. For all samples in this thesis ultracentrifugation has been used for isolation to facilitate direct comparisons with prior studies.

Although exosomes were first described in 1985 [48] there has been no consensus on the optimum purification strategy. The centrifugal force and the period of centrifugation differ widely between groups. The time required to pellet the exosomes is determined by the centrifugal force and the radius of the rotor used. These two properties are summarised by the k-factor. A lower k-factor suggests less time is required to sediment a particle. When sufficient information is reported to calculate the k-factor used in previous publications on exosomes it has varied from 356 [40] to 82 [10].

1.2. Proteomics

Since the success of the human genome project there has been a growing focus within the life sciences on studies at the system level. The goal has become the identification of the individual components that make up a system and their subsequent quantification under different conditions. Proteomics is the large scale determination of cellular function at the protein level. The completion of the human genome has been highly advantageous to the field of proteomics offering a sequence-based framework for data analysis [49]. Whereas the genome of an organism is constant throughout its existence the proteome is dynamic, as is the transcriptome, and varies between time-points, tissues, and environmental conditions. Importantly, although related, changes in the transcriptome do not always reflect changes in the proteome. As a result, to understand the proteome we must study it directly [50-52].

Proteomic studies can be broken down into two principle types; expression proteomics which focuses on analysing and quantifying the expression or more specifically concentration of expressed proteins within a cell, tissue or biofluid, and cell-map proteomics which focuses on understanding the interactions between proteins in the cellular context [53]. Recently the latter is increasingly referred to as interactomics [54]. In both cases the focus is on the identification and quantification of many proteins, or identification of many protein interactions, simultaneously.

The identification of many proteins in a complex mixture is possible using two main techniques. With the development of soft ionisation techniques mass spectrometry (MS), coupled with a variety of separation techniques, has

emerged as the leading approach for proteomics [54, 55]. MS based techniques require no prior knowledge of the proteins being studied and, as such, are ideally suited to discovery studies.

Protein arrays which offer a potential alternative for studying well characterised proteomes. Protein arrays, or protein microarrays, are conceptually similar to microarrays for transcriptomics. Instead of having a plate displaying a variety of short DNA fragments to which complementary cDNA molecules bind, a variety of capture probes are attached to a plate which then bind their complementary proteins [56]. For each protein to be assayed a capture probe, usually an antibody, must be developed which is highly sensitive, so that the target can be detected even at low concentrations, and highly specific, so that confidence can be maintained even in complex protein mixtures where cross-reactivity is a potential concern. For these reasons the creation of a protein assay capable of screening a sample for a large number of different proteins is expensive and technically challenging. Furthermore, good knowledge of the proteome being studied is a prerequisite for the development of protein arrays and they are better suited to validation studies rather than discovery studies. As no protein array currently exists for the exosomal proteome our focus is solely on mass spectrometry-based proteomics.

1.3. Mass Spectrometry Based Proteomics

The proteomic analysis itself can broadly be broken down into three steps; separation, ionisation and mass determination. With the exception of Surface Enhanced Laser Desorption Ionisation (SELDI) and Capillary Electrophoresis-Mass Spectrometry (CE-MS) - for which the aim is quantification without identification of the ions detected - the majority of proteome analyses include a fourth step; protein fragmentation by the action of an endopeptidase, usually trypsin. The identity of an ion cannot be accurately determined from the weight alone due to errors in mass determination, translational modifications and the complexity of the proteomes studied. Fragmentation of the protein and subsequent detection of several peptides provides greater confidence in the identity assigned [57]. For each step a variety of techniques have been developed, each with advantages and disadvantages. Although certain techniques are frequently used together (Table 1.3) with just a few exceptions they are interchangeable. It is important to use the combination of techniques which best match the characteristics of the experimental samples and the objectives of the study.

Technique	SELDI	CE-MS	2D PAGE MALDI	GeLC-MS/MS	LC-MS/MS
Separation method #1	Proteins in a sample selectively bind to treated areas on a chip.	Capillary electrophoresis of intact proteins	Isoelectric focusing of intact proteins	SDS-PAGE of intact proteins	Reverse phase liquid chromatography of tryptic peptides
Separation method #2			SDS-PAGE of intact proteins	Reverse phase liquid chromatography of tryptic peptides	
Digestion?	No	No	Yes	Yes	Yes
Mass spectrometer	Time of flight	Time of flight	Time of flight	Various though quadrupole and ion traps are popular	Various though quadrupole and ion traps are popular
Protein identification method	Protein identification is difficult. Focus is on quantitation of ions.	Protein identification is difficult. Focus is on quantitation of ions.	Mass fingerprinting or tandem mass spectra	Tandem mass spectra	Tandem mass spectra
Advantages	Can be automated High throughput	Can be automated High throughput	Uses widely available TOF instruments Quantitative	Protein identification Good separation	Protein identification Automatable
Disadvantages	Protein identification Variability	Protein identification Limited to <10 kDa	Labour intensive Resolution of small and membrane proteins	Not automatable	High abundance proteins mask proteins of lower abundance

Table 1.3 Summary of popular combinations of separation and detection methods used in mass spectrometry.

1.3.1. Separation

Due to the complexity of the proteome it is vital to separate it into fractions prior to analysis. This can be achieved 'online' with the mass spectrometer where the eluent from separation is fed directly into the mass spectrometer or 'offline' where the separation technique is not connected directly to the mass spectrometer.

When protein fragmentation by tryptic digestion is used, separation can occur at two distinct steps. Intact proteins can be separated prior to digestion or the peptides resulting from digestion can be separated prior to analysis on the mass spectrometer. Separation prior to and after digestion is not mutually exclusive with many studies combining both.

Two dimensional gel electrophoresis was first described in 1975 [58] and, despite alternative techniques being developed since, remains a mainstay of proteomic research. In 2D gel electrophoresis proteins are resolved in the first dimension by their isoelectric point in an electric field and then by their molecular weight using denaturing polyacrylamide gel electrophoresis in the second dimension. Proteins, resolved as spots on the gel, can be excised for identification.

2-D gels have several drawbacks. They perform poorly at resolving low molecular weight proteins, a particular problem for soluble urinary proteomics, and membrane proteins, a particular problem for exosome proteomics. Differences between gels also make comparisons difficult although the emergence of Difference In Gel Electrophoresis (DIGE) has minimised the difficulties with comparing result sets [59].

Liquid chromatography (LC) is the most commonly used 'online' fractionation method. There is a limit on the size of proteins which can be separated by liquid chromatography and so it is usually used following tryptic digestion. Typically reversed phase liquid chromatography is used in which the mobile phase is a polar aqueous solution and the stationary phase is hydrophobic, typically C_{18}. Elution of peptides is achieved by a water/acetonitrile gradient with an increasing acetonitrile concentration liberating increasingly hydrophobic peptides from the stationary phase. During this thesis LC has been used for all mass spectrometry experiments as it is well suited to separating the peptides or proteins entering the mass spectrometer sufficiently to enable the maximum number of ions to be analysed. Where possible separation of proteins on a denaturing polyacrylamide gel has been used to pre-fractionate the proteome prior to tryptic digestion.

1.3.2. Ionisation

A vital step in the development of mass spectrometry for proteomics was the development of 'soft' ionisation techniques. Prior to the development of these techniques getting a sample into the gas phase and ionising it caused the fragmentation of large biomolecules beyond the ability of mass spectrometers to analyse. The importance of viable ionisation strategies has been such that one half of the 2002 Nobel Prize in Chemistry was awarded to John Fenn [60] and Koichi Tanaka [61] for their work on electrospray ionisation (ESI) and matrix assisted laser desorption and ionisation (MALDI), respectively.

MALDI utilises a volatile matrix to lift peptides from a metallic surface upon excitation with laser light. MALDI requires a simple mixture of proteins to work. It can not be directly coupled to separation techniques and is difficult to automate so is labour intensive.

Since the development of ESI [62] the technique has since gone on to become the most popular method of ionisation for mass spectrometry instruments with the possible exception of time-of-flight mass spectrometers which are frequently used with MALDI. ESI is routinely used with 'online' separation techniques. The eluate from the separation is pumped into an electrically charged glass needle. At the tip the eluate is ejected as a charged droplet from which the solvent evaporates until the surface charge density is sufficient to desorb the solute ions [63]. The resulting ion is then taken in by the mass spectrometer for mass determination.

1.3.3. Protein Identification

Proteins are identified based on mass determination using mass spectrometry. The mass analysers most frequently used in proteomics are time-of-flight (TOF), the closely related ion trap (IT) and quadrupole (Q), and fourier transform ion cyclotron resonance (FTICR).

Protein identification follows two approaches; peptide mass fingerprinting and tandem MS. In peptide mass fingerprinting the observed peptide masses are compared with peptide masses calculated using an *in silico* digestion approach from protein or gene sequences in a database. The approach is limited in that relatively pure proteins or simple mixtures are required. The separation techniques to achieve this are often extensive which

limits the throughput of such techniques. Tandem MS combines the peptide mass data with the spectra generated by fragmenting the peptide ion in a collision cell. In theory this approach can be used to completely sequence the peptide. In practice analysis to this level is rarely used as it can be time-consuming and identifications with a high degree of confidence can be achieved based on *in silico* analysis of sequence databases [57, 64]. During this thesis when tryptic peptides have been analysed it has been on a tandem mass spectrometer enabling the use of the more robust tandem mass spectrometry approach (Figure 1.3).

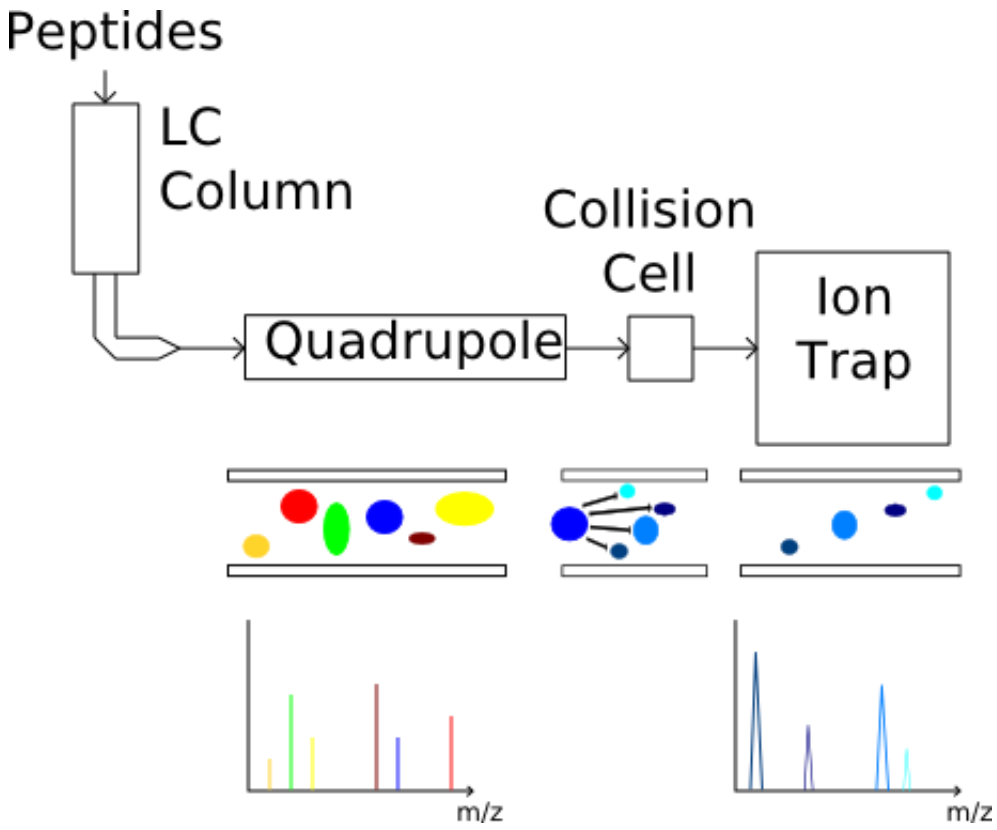


Figure 1.3 Schematic representation of a liquid chromatography tandem mass spectrometry instrument.
The five principal components are; the liquid chromatography (LC) column, the electrospray ionisation source, the primary mass spectrometer, the collision cell and the secondary mass spectrometer. Typically peptides are separated on the liquid chromatography column on the basis of hydrophobicity with more hydrophilic peptides eluting earlier. The eluate from the LC column connects directly to the electrospray ionisation source. The resulting stream of ions then enters the primary mass spectrometer which first surveys the m/z ratios of all the ions entering. When an ion of interest has been identified it then switches mode to selectively allow that ion to pass through into the collision cell. The ion then fragments and the resulting fragmentation ions enter the second mass spectrometer where their m/z ratios are measured. The combination of the parent ion m/z ratio and the m/z ratios of the fragmentation products is highly specific and enables the peptide to be identified.

1.4. Quantitative Mass Spectrometry

In addition to identifying proteins it would be advantageous to be able to also quantify them. Techniques for the quantitation of single or small panels of proteins in a sample are well established and include approaches such as western blot and Enzyme-Linked Immunosorbent Assays (ELISA) utilising specific antibodies against the protein of interest. In this thesis western blotting for specific proteins has been performed but there are limitations. Both ELISA and western blotting requires prior knowledge of the protein of interest and significant development time. Mass spectrometry has long been used for the quantitation of small molecules and is now increasingly being used for the quantitation of large panels of proteins in biological samples. To obtain robust quantitative data the peptide being quantified must be identified in all samples and must be unique for a particular protein. The adoption of mass spectrometry in this context is hampered as in some ways it is ill suited to quantitation. Although the spectra peak area corresponds to concentration the relationship is different for each compound or peptide.

Due to differences in the physicochemical properties of peptides their detectability within the mass spectrometer varies. For this reason it is difficult to compare two peptides or calculate their absolute concentration. Although absolute quantification would be advantageous, facilitating comparison between experimental data sets and laboratories, each peptide to be quantified must be synthesised so that it can be spiked into the sample at a known concentration. This process is laborious and rarely done for discovery based studies [65]. Using computational techniques progress has been made [64, 66-68] with quantitation to the correct order of magnitude

now becoming viable [68]. A more common approach is to consider only relative quantification utilising stable isotopes, chemical tags and label-free approaches (Figure 1.4). Despite significant differences in these approaches their performance is comparable. In a comparison of DIGE, ICAT and iTRAQ by *Wu et al* each technique was found to have different limitations but differences in accuracy were marginal [69]. Analysis of a dataset using both SILAC and label-free methods showed SILAC to perform better for low abundance proteins with additional data processing required to achieve comparable performance [70]. This study used a spectral counting approach to label-free analysis which is known to lose linearity at low abundances. A comparison of ICAT with label-free quantitation based on ion current area showed comparable performance between these techniques with spiked peptides detected at the 90% confidence interval for both approaches [71].

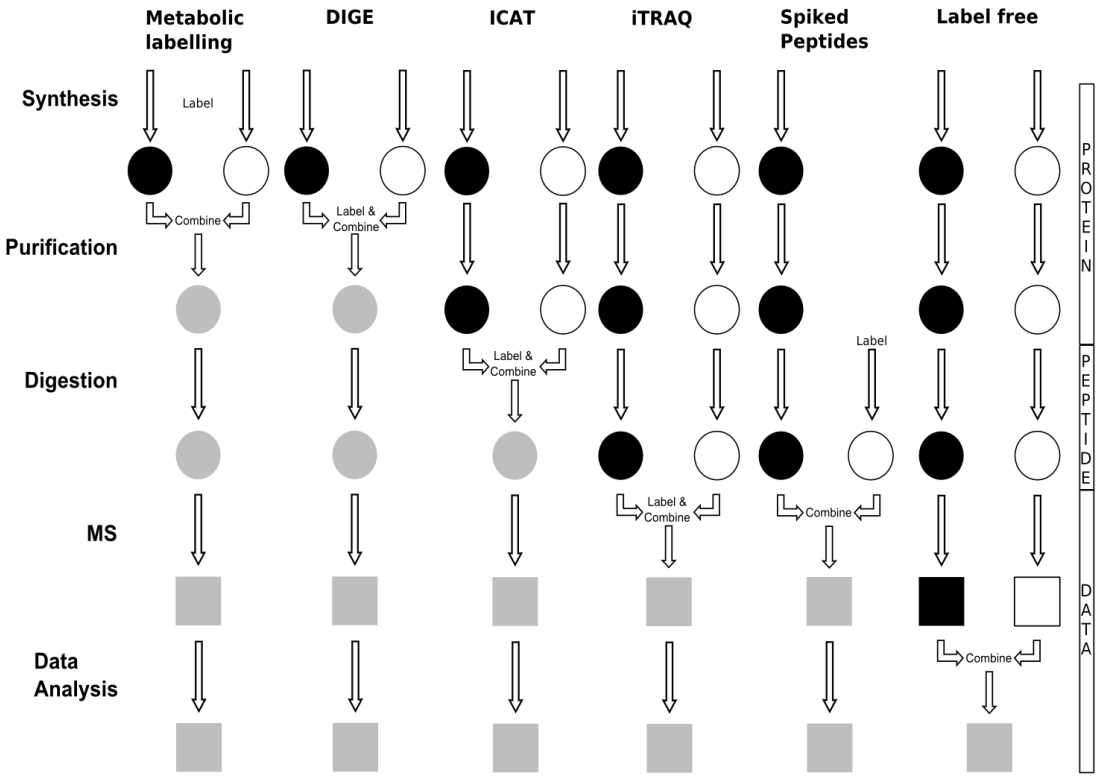


Figure 1.4 Flowchart representing the main approaches used for quantitative mass spectrometry.
Grey shapes represent pooled (black and white) samples. The labelling and combination steps in each approach are indicated.

1.4.1. Metabolic Labelling

The earliest opportunity a tag or label can be incorporated is during synthesis. To compare two samples a high percentage of a specific amino acid must include a heavy isotope in one of the samples and a light isotope in the other sample. Achieving this discrepancy in cell culture is relatively straight forward and inexpensive as the volume and mass of the system is relatively low. This technique is not generally thought to be suitable for larger intact organisms due to the prohibitive cost of the radioisotopes although variations to study tissues have been developed [72]. To reflect this limitation the technique is generally termed stable isotope labelling by amino acids in cell culture (SILAC) [73]. With different isotopes integrated into each sample they can be mixed and analysed on the mass spectrometer in the same run. This avoids inter-run variation. Due to the mass difference the peaks for the proteins in the two samples will be offset. The area under each of the peaks can be calculated and compared giving the relative concentration of the parent protein in the two samples. Although SILAC is not suitable for whole organism proteomics it is a good demonstration of the principals behind label based quantitative mass spectrometry. As it is not possible to integrate labels into the proteome of a whole organism during formation alternative strategies have been developed to integrate them after collection.

Of the techniques developed isotope-coded affinity tags (ICAT) and isobaric tag for relative and absolute quantitation (iTRAQ) have emerged as the most commonly used methods [74, 75]. Both these techniques involve additional experimental steps and potentially the inclusion of additional errors.

1.4.2. ICAT Labelling

The ICAT reagent consists of three elements: a thiol specific reactive group which attaches the tag to each cysteine residue in proteins; a linker which contains 8 positions filled with either hydrogen, in the light form, or deuterium, in the heavy form; and finally a biotin group which is used to isolate the tagged peptides. Two samples are labelled using different versions of the tag, mixed, digested with trypsin and then the labelled peptides isolated using the biotin group. As not all peptides will contain a cysteine residue this isolation step will significantly reduce the complexity of the sample and enable a more complete analysis of those that remain. The pooled samples are analysed on a mass spectrometer and the peptide peaks from each sample will be slightly offset due to the 8 Da difference in the mass of the tag [76].

ICAT labelling occurs at the earliest point after collection which should minimise the effect of errors introduced during sample processing. As the ICAT label reacts with cysteine, only proteins containing this amino acid can be quantified.

1.4.3. iTRAQ

The iTRAQ system utilises an amine specific reactive group to label all peptides in a sample. Unlike ICAT where the intact protein is labelled, the iTRAQ labelling step occurs after protein digestion. The amine reactivity and labelling post digestion should ensure that all peptides are labelled regardless of their composition. As with ICAT quantitation, iTRAQ utilises stable isotopes but unlike ICAT the mass of the intact tag does not change (Figure 1.5). This avoids issues with chromatographic separation. This isobaric tag, in addition to the reactive group, contains two parts; a reporter group and a balance group. Although the mass of each of these groups varies over the ranges 114.1-117.1 for the reporter and 28-31 for the balance group (there are four tags available with the classical iTRAQ system and now eight tags [77, 78] with a revised and expanded version rather than the two available with ICAT and most other labelling strategies) the overall mass of the two groups combined is kept constant using differential isotopic enrichment. For example the 114.1 Da reporter group is exclusively attached to the 31 Da balance group and the 117.1 reporter group is attached to the 28 Da balance group giving a consistent mass for the combined group of 145.1 Da. In the initial mass spectrum the peptides labelled with the four different tags all have the same m/z ratio and so appear as a single unresolved peak. Collision induced dissociation (CID) liberates the reporter group which, due to the difference in their masses resolve as discrete peaks enabling, by comparison of their intensities, the relative concentrations of their original peptides to be deduced. The balance group is lost without charge and the fragments of the peptide originating from each sample are isobaric and so their ion signals are cumulative and reinforce the signal obtainable from any one sample analysed alone [79].

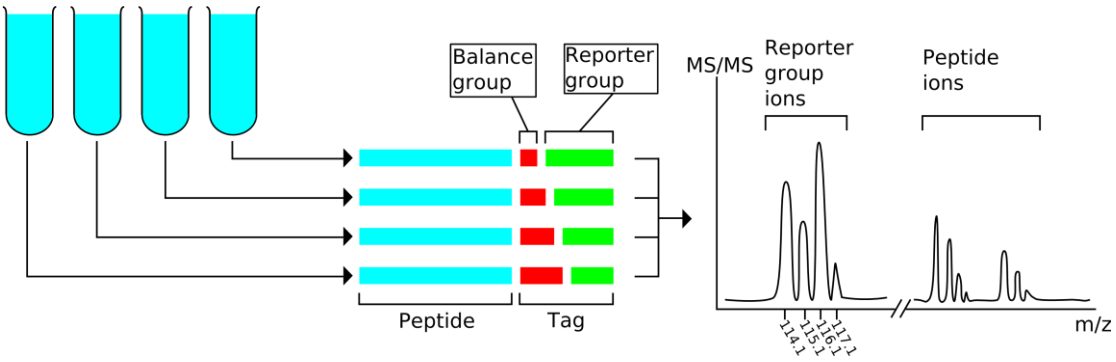


Figure 1.5 Schematic representation of the 4-plex version of isobaric tag for relative and absolute quantification (iTRAQ) quantitative mass spectrometry. Tryptic digests from four experimental conditions are labelled with isobaric tags. The mass of the reporter group in each tag varies but is compensated for by a change in the mass of a balance group so that the mass of the overall tag remains constant. As the peptide passes through the first mass spectrometer the peptides appear identical. Following fragmentation in the collision cell the reporter ions are liberated and are quantified in the second mass spectrometer.

1.4.4. Label-free Quantitation

Both ICAT and iTRAQ function because the spectral peak area of a species is proportional to the concentration of the measured species whether that species is a label or a peptide-label conjugate. This relationship holds true for unlabelled peptides as well. Thus, in theory at least, proteins can be quantified, relative to the same protein in other samples using mass spectrometry without labelling. In the context of LC-MS/MS an ion chromatogram is extracted from the LC-MS/MS run and the peak area for a species integrated over the chromatographic timescale. The area or intensity of the peak is then calculated and can be compared between runs. The advantages include simplified sample preparation and the ability to compare an unlimited number of runs. For comparison, ICAT is limited to directly comparing two samples [76] and iTRAQ to four [79] or eight [77, 78]. The disadvantage is that high reproducibility between runs is crucial. This is not trivial with peptide elution times varying even between consecutive runs. Software has been developed to synchronize LC chromatograms between runs [80] but this is not an ideal solution and the possibility for errors remains and is less well understood than for labelling approaches such as ICAT and iTRAQ.

With label-free quantitative LC-MS/MS it is important to balance the acquisition of survey spectra for quantitation and fragment spectra for identification. LC supplies a near constant stream of new ions for analysis to the mass spectrometer. For complex samples new ions may be supplied to the mass spectrometer faster than it can analyse them. As a result some species will be skipped from the analysis. The added time required to obtain detailed survey spectra in addition to the fragmentation spectra exacerbates

this problem. This has led several groups to analyse each sample twice; once to obtain as many fragmentation spectra as possible for protein identification and a second time to obtain as many survey spectra as possible to ensure multiple samplings of the chromatographic peaks and accurate quantitation.

1.4.5. Peptide Spiking

So far the methods discussed have focused on relative quantitation between samples. For the purposes of biomarker discovery relative quantitation between samples will likely be sufficient. If absolute quantitation is required then the approach taken has been to synthesise peptides matching proteins of interest and spike them into samples. As the concentration of these spiked peptides is known, comparison against them allows absolute quantitation. This approach means that you need to know in advance the proteins you want to quantify and requires significant preparation but remains the gold standard for absolute protein quantification. This situation is similar to that for protein arrays and, as for protein arrays, peptide spiking is more suited to validation studies than discovery studies.

1.5. Biomarkers

With the development of techniques for the large scale identification of proteins, interest has grown in studying tissues both directly and also indirectly via the body fluids. Although much of the work within proteomics has focused on cataloguing what is present there has been growing interest in the use of proteomic techniques for biomarker discovery. The National Institutes of Health (NIH) [81] has defined a biomarker as, "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." Within the context of proteomics the measured characteristic would be a protein. The aim has, and continues to be, the identification of novel, hopefully superior, markers of disease enabling more timely and effective treatment. Kidney and neurological disease are two areas where improved methods of detection and monitoring are particularly needed.

1.5.1. Urine, biomarkers & AKI

For many kidney diseases non-invasive diagnosis remains a significant challenge. A biopsy is commonly required for an accurate diagnosis but this is an inherently invasive procedure. The risk of an adverse event (such as haemorrhage) is increased by a variety of factors including obesity, severe hypertension and end stage renal disease (ESRD). Although a biopsy is rarely indicated in ESRD [82, 83] the need to control hypertension may delay biopsy. Well characterised biomarkers detectable in the urine would provide safe, repeatable alternatives.

Acute kidney injury (AKI) is a serious medical condition which may occur following a variety of insults and is known to complicate treatment and have an adverse effect on mortality [84]. Around 19% of patients with moderate sepsis and 23% of patients with severe sepsis [85] will go on to develop AKI increasing mortality from 25-30% to 40-70% [86]. AKI is difficult to treat and early aggressive management is the principal approach taken. As such, early diagnosis is important. Although several potentially superior new biomarkers are undergoing late-stage clinical trials, currently AKI is diagnosed by observing increases in plasma creatinine and decreases in urine flow rates [84, 87]. Plasma creatinine is a function of creatinine generation, the volume of distribution and creatinine excretion. Following a decrease in excretion it can be 48 hours before a change in plasma creatinine becomes apparent and 7 days before the extent of the change is known [84]. This assumes that the rate of production and volume remain constant which, particularly in conditions such as sepsis, will not necessarily be correct[88]. Treatment and management of AKI will be most successful if started soon after injury. Identifying novel biomarkers capable of identifying AKI before plasma creatinine changes has therefore been a priority.

Several new proteins have been shown capable of detecting AKI before changes become apparent in plasma creatinine [89-92]. For example N-acetyl-β-glucosaminidase (NAG), cystatin C, kidney injury molecule (KIM)-1, neutrophil gelatinase-associated lipocalin (NGAL) and IL-18 have each received considerable interest [89]. They have been found to increase as soon as 1 hour after injury [93] and up to 4 days before injury would be detected using serum creatinine [94]. NAG is a proximal tubule lysosomal enzyme. It is elevated in the urine following tubular injury 12-96 hours prior to serum

creatinine although it can be elevated in some unrelated conditions [94]. Cystatin C is a cysteine protease inhibitor which is produced by all nucleated cells and then filtered by the glomeruli before reabsorption by the tubule [89]. In this respect it is the novel biomarker most similar to serum creatinine. In comparison to serum creatinine detection of AKI is possible 1-2 days earlier with urinary cystatin C [95]. KIM 1 is a type I glycoprotein containing immunoglobulin-like and mucin domains. KIM 1 mRNA levels increased more than any other gene in a rodent model of kidney injury [96]. KIM 1 is expressed on the apical membrane ciliae of the proximal tubule where the ectodomain is shed into the lumen. Urinary KIM 1 responds to injury more slowly than NGAL or IL-18 but is still elevated 12 hours after ischaemic insult [92]. NGAL was identified as one of several proteins increased more than 10-fold within hours of an ischaemic injury [93]. Via a siderophore NGAL binds iron ions and has been shown to be protective against ischaemic kidney injury [97]. Like NAG, it can be elevated in response to some conditions unrelated to AKI [89]. IL-18 is a pro-inflammatory cytokine upregulated in the urine in response to ischaemia, inflammatory nephritis and nephrotoxicity. An elevation in IL-18 can precede serum creatinine by 1-2 days [98]. Our understanding of AKI, although improved in recent years [99], is still far from complete and for this new generation of potential biomarkers there are still uncertainties. Additional study is required on these biomarkers and AKI in general to further improve diagnosis and treatment.

The plasma [100], serum, ascites fluids [101, 102], amongst other biofluids together with tissue biopsies have all been investigated using proteomic techniques. The urine has several advantages in certain situations [103]. The

collection of urine is non-invasive, straightforward and it is available in large volumes from almost all patients. Approximately 70% of the urinary proteome originates from the kidneys. The remaining 30% being filtered from the circulation [104]. Therefore the urine is well-suited for the discovery studies designed to identify new biomarkers of kidney disease.

Studying the urinary proteome also presents difficulties; the proteome varies diurnally within an individual, the protein concentration is relatively low and again may vary, and the urine contains a variety of interfering compounds including salts and organic compounds [104].

1.5.2. CSF and Neurological Disease

Neurological diseases are a significant cause of morbidity and mortality. In Europe, 23% of lost healthy years of life and 50% of years lived with disability are caused by neurological diseases [105]. The situation may worsen in the decades ahead because the incidence of Alzheimer's disease, the commonest cause for dementia, is set to quadruple by 2050 [106]. Diagnosis of neurological disease is often difficult, for example, despite intensive interest in biomarkers for Alzheimer's disease over recent years we are still unable to monitor many aspects of disease progression [107]. In addition to ensuring that patients receive the correct care, reliable biomarkers able to track disease progression would significantly aid clinical trials for new therapies by providing surrogate endpoints.

A biomarker present in the blood would be ideal due to the relative ease of collection but discovery is challenging. The blood-brain barrier limits the flow of proteins from the central nervous system into the circulation.

Proteins which do reach the circulation must then be detected against the background of proteins released by other organs of the body and high abundant proteins such as albumin. Given the difficulties with plasma proteomics and the high human and financial cost of neurological disease a growing number of publications have studied the proteome of the cerebrospinal fluid in search of biomarkers [108, 109]. Although potential candidates for a variety of diseases have been identified there remains a pressing need for further study.

1.6. Concluding Remarks

We hypothesise that exosomes reflect the internal state of their cells of origin. We have explored this hypothesis via four distinct aims

1. Development and validation of an exosome purification protocol in the urine.
2. Investigate whether changes in exosomal protein faithfully reflect changes in the cell and whether the exosomes can transfer proteins between cells.
3. Investigate whether exosomes are present in human CSF.
4. Catalogue and assess the variability in the proteome of the CSF exosomes.

2. Materials and Methods

2.1. Biofluid preparation

The protocol used for the collection and storage of a biofluid has an impact on the recovery of exosomes [110]. For the three fluids studied as part of this thesis the established protocols for exosome recovery from urine have been followed [110, 111]. Studies have demonstrated that protease inhibitors are not required for exosome isolation from human urine [112] and, as they may interfere with the proteomic analysis, they were omitted for these studies. For studies on tissue culture media and the CSF, where it is not yet clear whether protease inhibitors can be safely omitted, they have been used.

The protease inhibitors used were leupeptin (Sigma, cat #L9783) and phenylmethylsulfonyl fluoride (PMSF; Sigma cat #P7626). Leupeptin is a small peptide which binds to the active site of serine and cysteine proteases including plasmin, trypsin, papain and cathepsin B. PMSF is a non-competitive inhibitor which selectively forms a covalent bond with the active site serine in serine proteases. It is highly effective but quickly degrades in aqueous solution. For all samples sodium azide was added as a bacteriostatic agent. By binding irreversibly to the heme cofactor of cytochrome oxidase in gram negative bacteria sodium azide effectively inhibits growth.

2.1.1. Urine

Urine was collected from healthy individuals, fellow students within the BHF/University Centre for Cardiovascular Science and from patients with sepsis. Samples were obtained with informed consent and full ethical approval. If not immediately processed the urine samples were stored at -80°C. When ready to be processed the urine samples were thawed with

vigorous vortexing when the sample was partially thawed and again when fully thawed [110]. After thawing the samples were kept on ice or stored at 4 °C throughout processing. Sodium azide (final concentration 3.34 mM) was added to samples once thawed as a preservative.

2.1.2. CSF

CSF was collected from patients undergoing thoraco-abdominal aortic aneurysm repair (with full ethical approval). This group was chosen as they have a CSF drain inserted peri-operatively as part of routine clinical management [113, 114]. Samples were aliquoted then stored at -80 °C until processing. Samples were thawed with vigorous vortexing when the sample was partially frozen and again when fully thawed. Protease inhibitors and preservative (final concentration: 3.34 mM sodium azide, 0.5 mM PMSF, 20 μM Leupeptin) was added to the collected CSF.

2.1.3. Media

Cell culture media was collected and immediately frozen at -80 °C until processing. Samples were thawed with vigorous vortexing when the sample was partially frozen and again when fully thawed. Protease inhibitors and preservative (final concentration: 3.34 mM sodium azide, 0.5 mM PMSF, 20 μM Leupeptin) was added to the collected media.

2.2. Exosome purification

The biofluid samples were centrifuged at 15,000 x *g* for 10 minutes to pellet any shed cells, large membrane fragments and other debris. The supernatant was then centrifuged at 200,000 x *g* for 60 minutes to pellet the low density membrane fraction. The ultracentrifugation was performed using a Beckman Optima MAX-E ultracentrifuge and a MLA-80 rotor. The k-factor for this rotor at 200,000 x *g* was 65. The pellet was resuspended in phosphate buffered saline (PBS) (137 mM sodium chloride, 12mM sodium phosphate, 2.7 mM potassium chloride; pH 7.4) and then centrifuged again at 200,000 x *g* for 60 minutes before resuspension again in PBS. All centrifugations were performed at 4 °C. The protein content of the low density membrane fraction was determined using the Bicinchoninic acid (BCA) protein assay kit (Pierce Cat # 23227) following the manufacturer's instructions.

2.3. Protein Separation

Electrophoresis separates compounds based on their ability to pass through a medium. Denaturing polyacrylamide gel electrophoresis (PAGE) separates proteins based on their size. The technique is used in conjunction with stains to visualise the molecular weight of the proteins present in a sample and as a preliminary step in western blots.

2.3.1. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Low density membrane samples were solubilised with 5x Laemmli sample buffer (0.5 M dithiothreitol (DTT), 10% sodium dodecyl sulphate (SDS), 14% sucrose, 0.01% bromophenol blue) and separated on a 1D SDS-PAGE gel. Pre-cast 12% gels from Bio-rad (cat # 1611102) and Thermo (cat # 25202) were used for the SDS-PAGE. The buffer systems for these gels were different; the Bio-rad gels used a 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS)-Glycine system (25 mM TRIS, 192 mM Glycine, 0.1% SDS, pH 8.3), the Thermo gels used a TRIS - 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) system (Thermo 20X Tris-HEPES buffer, cat # 28368). The Bio-rad gels were run at 25 mA for 1 hour. The Thermo gels were run at 100V for 45 minutes. For both gel systems the Invitrogen SeeBlue Plus2 pre-stained standard (cat # LC5925) was used.

During evaluation of the Agilent MARS column (see below), samples were separated using the Invitrogen NuPAGE Novex Bis-Tris 12% Mini Gel system (cat # NP0342BOX). Samples were prepared following the manufacturer's instructions with 4x lithium dodecyl sulphate (LDS) Sample Buffer (cat # NP0007) and 10x Sample Reducing Agent (cat # NP0004). The

gels were run at 200V for 35 minutes using the 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (cat # NP0002). The Invitrogen SeeBlue pre-stained standard was used (cat # LC5625) with this gel system.

2.3.2. Colloidal Coomassie Blue staining

The molecular weight distribution of protein in a sample was assessed using a Coomassie stain [115]. The gel was fixed in a 7% glacial acetic acid, 40% methanol solution for 1 hour prior to the gel being stained using a Brilliant Blue G – colloidal concentrate (Sigma Aldrich, cat # B2025) stain following the manufacturer's instructions. The gel was briefly washed in a 25% methanol solution following which the gel was photographed. Destaining was achieved with a 10% glacial acetic acid, 40% methanol solution.

2.3.3. Coomassie blue staining

During assessment of the Agilent MARS column the presence of protein in a sample was verified by running a small aliquot on a denaturing polyacrylamide gel and staining with a Coomassie based stain. The gel was washed in deionised water three times for a total of 15 minutes and then GelCode Blue Safe Stain (Thermo Scientific, cat # 24594) added for 30-40 minutes. The gel was then destained in deionised water to visualise the protein. All steps were performed on an orbital shaker.

2.3.4. Methanol/Chloroform Protein Precipitation

A methanol / chloroform protein precipitation was used when concentrated samples were required for electrophoresis. To 1 part sample, 4 parts methanol was added and the sample vortexed until mixed. Then 1 part

chloroform was added, followed by vortexing, and then 3 parts distilled water and the sample again vortexed. The sample was then centrifuged for 5 minutes at 15000 x *g*. Centrifugation separates the organic phase containing the chloroform from the aqueous phase. The protein is concentrated at the liquid interface. The aqueous top layer is discarded with care taken to avoid disturbing the interface and 4 parts methanol added to the organic phase and the protein at the interface. The sample is then vortexed and centrifuged at 15000 x *g* for 30 minutes. The supernatant is then removed and the precipitate resuspended in fresh buffer. All centrifugation steps are performed at 4 °C and the sample stored on ice.

2.4. Western blot

Denaturing polyacrylamide gel electrophoresis was used to separate proteins based on molecular weight. Following electrophoresis, the proteins were transferred to a PVDF membrane (Invitrogen cat# LC2002) using a 200 mA current for 1 hour. The membrane was blocked with 5% fat-free milk in TBST (137 mM sodium chloride, 20 mM TRIS, 0.1% Tween 20; pH 7.5) and then probed with antibody to the protein of interest (Table 2.1). The membrane was washed and then probed with an appropriate secondary antibody conjugated to horseradish peroxidase before visualisation using ECL western blotting analysis system (Amersham cat# RPN2109) and Thermo CL-XPosure film (cat # 34090).

Target Protein	**Target Species**	**Company**	**Catalog number**	**Host species**	**Conjugation**
Flotillin 1	Mouse, Rat, Human, Chicken	BD Biosciences	610820	Mouse	-
TSG101	Human, Mouse, Rat	Abcam	Ab83	Mouse	-
CD24	Human	A kind gift from Peter Altevogt, German Cancer Research Center, Heidelberg		Mouse	-
cJun	Human, Mouse, Rat, *Xenopus*	Santa Cruz	Sc-1694	Rabbit	-
Fetuin A	Mouse	R&D Systems	MAB1563	Rat	-
AQP2	Human, Mouse, Rat	Millipore	AB3274	Rabbit	-
IgG	Human	AbD Serotec	STAR90	Goat	-
IgG1 Isotype control		BD Pharmingen	554121	Mouse	-
IgG	Rabbit	Santa Cruz	Sc-2004	Goat	HRP

Target Protein	Target Species	Company	Catalog number	Host species	Conjugation
IgG	Mouse	Santa Cruz	Sc-2005	Goat	HRP
IgG	Rat	R&D Systems	HAF005	Goat	HRP
IgG	Mouse	Sigma	G7652	Goat	10nm Gold particle
IgG	Rabbit	Sigma	G3779	Goat	10nm Gold particle

Figure 2.1 Antibodies used for western blot and Immuno-TEM.

2.4.1. Membrane Stripping

Membrane stripping removes the antibodies used to detect a protein allowing the membrane to be probed with a different antibody. This approach enables more information to be collected from a given amount of experimental sample.

The PVDF membrane was washed 3 times in TBST for 2 minutes and then incubated in stripping buffer (0.1% SDS, 1% Tween-20, 0.2 M Glycine, pH 2.2) for 10 minutes. The buffer was then removed and replaced with fresh stripping buffer for a further 10 minutes after which it was again removed and replaced with TBST. The membrane was washed 3 times with TBST for 5 minutes. To confirm that the attached antibodies had been successfully removed the membrane was treated with ECL reagent and then a photographic film was exposed to the membrane for 30 minutes. On developing, the film should be clear. The membrane was washed a further 3 times with TBST before being reprobed.

2.4.2. Ponceau S Staining

Ponceau S staining was used to visualise the proteins adhering to the PVDF membranes following transfer. The membrane was washed 3 times in distilled water for 5 minutes and then stained with Ponceau S solution (Fluka cat # 81462) for 5 minutes. The membrane was then washed again 3 times in distilled water for 5 minutes to remove background staining. The membrane was photographed and then washed with distilled water before destaining using 0.1 N NaOH for 30 seconds. The membrane was then washed with distilled water 3 times for 2 minutes and then with TBST 3 times for 2 minutes.

2.5. Isopycnic Centrifugation

Exosomes have been shown from previous reports to have a characteristic density of 1.10 – 1.15 $g.cm^{-3}$ [23, 40]. Using isopycnic centrifugation it is possible to separate particles based on their density. A variety of solutes can be used to establish a density gradient through which a particle will migrate until the density of the surrounding solution matches its own density. Once equilibrium has been reached centrifugation can stop and fractions collected of known density. Sucrose has widely been used as the solute of choice for density gradients for the isolation of exosomes. Changing the solute can subtly alter the density to which a particle localises and so sucrose was used to allow consistency with prior studies.

The resuspended low density membrane fraction was diluted into the top fraction of a step gradient comprising layers of 2, 1.3, 1.16, 0.8, 0.5 and 0.25 M sucrose in a Beckman ultraClear tube (cat # 347356). The gradients were centrifuged for 2.5 hours at 200,000 x *g* in a Beckman TLS-55 rotor. Six fractions were collected from the gradient and stored for density determination on an Anton Paar DMA 35N density meter and western blot analysis.

2.6. Visualisation of Exosomes

Exosomes have a diameter of 20 – 100 nm and a distinctive shape [10, 40, 116]. Visualising structure at this scale is not possible with standard light microscopy due to diffraction limits using wavelengths visible to the human eye. Electron microscopy, due to the shorter wavelength of the electrons, can visualise structures at this scale. The protocol followed is based on several previously published [10, 116] with variations made to better match the equipment available.

2.6.1. Transmission Electron Microscopy (TEM)

Following isolation of the exosomes using ultracentrifugation the resuspended sample was mixed 1:1 with 4% paraformaldehyde. A drop of this solution was placed on a Petri dish and then a formvar coated 200 mesh gold grid (Taab cat# F218/G025) floated on top for 20 minutes. All subsequent steps are performed in the same way. The grid was moved to PBS for a two 5 minute washes. Then the exosomes were re-fixed on the grid using a 1% glutaraldehyde solution. The grid was again washed twice in distilled water for 5 minutes. Finally the grid was transferred to a drop of 0.5% uranyl acetate / 2% 25 centipoise methyl cellulose (Sigma Aldrich cat #M6385). Following staining for 5 minutes excess fluid was removed, the grid allowed to air dry and then examined on a Phillips CM120 BioTwin transmission electron microscope.

2.6.2. Immuno-TEM

The low density membrane fraction was mixed 1:1 with 4% paraformaldehyde. A drop of this solution was placed on a Petri dish and

then a formvar coated 200 mesh gold grid (Taab cat# F218/G025) floated on top for 20 minutes. All subsequent steps were performed in the same way with washing between each step. The grid was blocked for 5 minutes with 0.05 M Glycine / PBS then 1% BSA / PBS, then transferred to a 1:200 dilution of the antibody of interest or appropriate isotype control in 0.02% Triton X-100 PBS for 45 minutes at room temperature. Then the grid was incubated with a 1:40 dilution of a 10 nm gold conjugated anti-mouse IgG antibody for 60 minutes at room temperature. Finally the grid was incubated with 1% glutaraldehyde for 5 minutes, then contrasted and embedded with 0.5% uranyl acetate / 2% methyl cellulose. Excess fluid was removed, the grid allowed to air dry and then examined on a Phillips CM120 BioTwin transmission electron microscope.

2.7. Protein digestion using trypsin

Tandem mass spectrometric analysis of tryptic digests provides more robust identification of proteins than mass spectrometric analysis of intact proteins. Protein digestion with trypsin can be performed in a number of ways with the optimal approach dependent on the experimental design and the sample being studied.

2.7.1. In-gel tryptic digestion

The SDS-PAGE gel was sliced into ten sections and then each slice was cut down to gel cubes approximately 1mm in size. These gel pieces were then washed in distilled water for 15 minutes. The distilled water was removed and 50% acetonitrile added for a further 15 minutes. This was then removed and replaced with fresh 50% acetonitrile. The solution was then replaced with 100 mM ammonium bicarbonate for 5 minutes. Then an equal volume of acetonitrile was added for a 15 minute incubation. The solution was then removed and the gel pieces dried in a speed vacuum at 60 °C for 45 minutes.

The cysteine residues in the proteins were then reduced and alkylated. A 10 mM DTT / 100 mM ammonium bicarbonate solution was added to the gel pieces and they were then incubated at 56 °C for 45 minutes without agitation. The samples were allowed to cool and then an equal volume of 55 mM iodoacetamide / 100 mM ammonium bicarbonate was added and the gel pieces incubated in the dark for 30 minutes. The solution was removed and replaced with 100 mM ammonium bicarbonate. Following incubation for 5 minutes, an equal volume of acetonitrile was added and incubation

continued for 15 minutes. The solution was removed and the gel pieces dried in a speed vacuum.

The proteins were then digested by adding a sufficient volume of 0.1 μg/μl sequencing grade modified trypsin (Promega cat #V5111) to re-hydrate the gel pieces. The gel pieces were incubated on ice for 45 minutes after which any excess solution was removed and sufficient buffer solution added to cover the gel pieces which were then incubated at 37 °C overnight without agitation. The following morning the solution was removed and stored on ice. Ammonium bicarbonate (25 mM) was then added to the gel pieces and incubated for 15 minutes. An equal volume of acetonitrile was then added and again incubated for 15 minutes. This solution was then removed and pooled with the overnight supernatant. Formic acid (5%) was then added to the gel pieces and incubated for 15 minutes before an equal volume of acetonitrile was added and again incubated for 15 minutes. The solution was removed and pooled with the previous supernatant and the gel pieces incubated with 5% formic acid and then acetonitrile as before. The solution was again removed and pooled with the previous supernatants and then 10 mM DTT was added to give a final concentration of 1 mM DTT. The supernatant was then completely dried in a speed vacuum at 60 °C and the residue stored at -80 °C until processing on the mass spectrometer.

2.7.2. In-solution digestion

Following purification using methanol / chloroform precipitation the proteins were resuspended in 50 mM ammonium bicarbonate solution to a concentration of 12 pmol/μl calculated on the assumption that the mean molecular weight of a protein is 50 kDa. The proteins were then reduced by

incubation at 50 °C for 15 minutes with a 50 fold molar excess of DTT. To prevent the disulphide bonds reforming the cysteine and methionine residues were capped with alkyl groups to form stable derivatives by incubation at room temperature with a 2 fold molar excess of iodoacetamide to DTT. Sequencing grade modified trypsin (Promega cat # V5111) was reconstituted in the supplied resuspension buffer and then added to the protein sample to a ratio of 1 part protease to 50 parts protein by weight. The samples were then incubated at 37 °C overnight. The enzymatic reaction was stopped by the addition of trifluoroacetic acid to a final concentration of 10%.

2.7.3. On-filter digestion

Sample was loaded on the molecular filter which was centrifuged at 12000 x *g* for 45 minutes, the molecular filter tube was refilled with 100 mM ammonium bicarbonate and the filter again centrifuged for 45 minutes. DTT was then added to the tube at a 50 fold molar excess to the estimated protein present and the tube incubated at 50 °C for 15 minutes. Iodoacetamide was then added at a 2 fold molar excess to the DTT and the tube kept in the dark for 15 minutes at room temperature. Sequencing grade modified trypsin (Promega cat #V5111) was then added at 20 fold less than the estimated protein present. The tube was incubated overnight at 37 °C and then centrifuged at 12000 x *g* for 45 minutes with the eluate collected. Ammonium bicarbonate (50 µl 100 mM) was added to the filter which was again centrifuged and the eluate collected. Finally 50 µl of 10% acetonitrile was added and the filter again centrifuged with the eluate collected. The collected eluates were pooled and trifluoroacetic acid was added to a final concentration of 10%.

2.7.4. Quantitative MS on spiked samples

Label-free quantitative mass spectrometry was performed on samples spiked with peptides following digestion and spiked with proteins prior to digestion in two separate experiments. For the first experiment a sample of purified urinary exosomes was run briefly on a SDS-PAGE gel and the proteins digested and extracted following the in-gel digestion protocol. The resulting peptides were then split into two aliquots. Each aliquot was spiked with 5 peptides to represent 1% and 4% of total protein by mass. These peptides, all obtained from Sigma-Aldrich, were Angiotensin II acetate (Cat# A9525), Osteocalcin fragment 7-19 (Cat# O3632), Gly-Glu-Gln-Arg-Lys-Asp-Val-Tyr-Val-Gln (Cat# G3774), Chromostatin-20 (Cat# C1680) and Chromogranin A (Cat# C6446). These peptides were chosen because they had been used in a previous peptide spiking study [117]. The two aliquots were then analysed using LC-MS/MS. The peaks corresponding to the spiked peptides were then located in the spectra using the DataAnalysis software and the area of the peaks compared between the samples.

For the second experiment a sample of urinary exosomes was split into two aliquots and then spiked with the SDS-PAGE low range molecular weight standard (Bio-Rad cat# 161-0304) containing six proteins (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme) to represent 1% and 4% of total protein by mass. Each aliquot was then briefly run on an SDS-PAGE gel before the proteins were digested with trypsin and extracted from the gel following the in-gel digestion protocol. The two aliquots of extracted peptides were then analysed by LC-MS/MS. The peptides derived from the spiked proteins were identified by querying the MASCOT software. The peak area for each of the identified peptides was

measured using the DataAnalysis software and then compared between the two aliquots.

2.8. Immunoglobulin depletion

2.8.1. Immunoglobulin depletion using Protein G

The purified exosomes were mixed with 1.25 mg Protein G immobilized on cross-linked 4% agarose beads (Fluka cat #83219) and incubated at room temperature for 10 minutes. The agarose beads were pelleted by centrifugation at 2000 x *g* for 5 minutes and the supernatant collected. The beads were then washed with PBS solution, centrifuged again and the supernatants pooled.

2.8.2. Immunoglobulin depletion using Agilent Multiple Affinity Removal System (MARS) column

The proteins in the purified exosomes were extracted using a methanol/chloroform precipitation and then resuspended in an 8M urea solution which was immediately diluted to 4M. The Agilent MARS column is sensitive to organic solvents and a urea solution is used to elute bound proteins from the column. For the column to function correctly it is therefore necessary to replace the buffer. This was achieved by dilution into the sample buffer for the MARS column and subsequently concentrating the sample on a Microcon Ultracel YM-3 molecular filter (Millipore cat #42403). The filter loaded with the sample was centrifuged at 12000 x *g* for 45 minutes, the sample again diluted with sample buffer and the filter centrifuged again. In total this process of dilution and centrifugation was repeated three times. The sample was then run on the MARS column following the manufacturer's instructions and the low abundance and high abundance protein fractions collected. The collected volume was approximately 400 µl for the low

abundance proteins and 1.5 ml for the high abundance proteins. These samples were then concentrated on molecular filters prior to analysis.

2.9. Mass Spectrometry

Two different types of mass spectrometers were used based on the type of sample being analysed.

2.9.1. GeLC-MS/MS

An aliquot of resuspended exosomes were run on a Bio-Rad SDS-PAGE gel to fractionate the proteome. The gel was then stained with colloidal coomassie blue stain (Sigma-Aldrich Cat# B2025-IEA) to visualise the proteins and the protocol for in-gel tryptic digestion followed. The resuspended tryptic peptides were then separated on a Dionex UltiMate 3000 series HPLC instrument with a Dionex Pepmap100 75 μm by 15 cm column with a 300 μm by 5 mm trap column maintained at a constant temperature of 40 °C. The loaded peptides were eluted from the column by an acetonitrile solution which increased in concentration from 5% to 72% over 35 minutes. The eluate was connected to the ESI source on a Bruker Daltonics HCT mass spectrometer.

2.9.2. LC-MS/MS

In addition to GeLC-MS/MS the proteome of the CSF derived exosomes was also investigated using LC-MS/MS. This was done in preparation for a quantitative analysis for which separation on a gel can negatively impact reproducibility. The proteins present in the CSF exosomes were separated from the lipid components using a methanol / chloroform precipitation. A SDS-PAGE gel was then run on a small aliquot to verify the presence of protein and then an in-solution digest was used to generate tryptic peptides. The resuspended tryptic peptides were separated on a Dionex UltiMate 3000

series HPLC instrument with a Dionex Pepmap100 75 μm by 15 cm column with a 300 μm by 5 mm trap column maintained at a constant temperature of 40 °C. The loaded peptides were eluted from the column by an acetonitrile solution which increased in concentration from 5% to 72% over 35 minutes. The eluate was connected to the ESI source on a Bruker Daltonics HCT mass spectrometer.

2.9.3. LC-FTICR whole protein MS

Following resuspension of the methanol / chloroform precipitation pellet, the proteins were separated on a Dionex UltiMate 3000 series HPLC instrument. The eluate was connected to the ESI source on a Bruker Daltonics 12T SolariX Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR MS)

2.10. Cell culture

To study exosome release and also assess the biological role of exosomes an *in vitro* cell model has been used. Murine cortical collecting duct (mCCD) cells [118] were used as a model kidney cell line.

2.10.1. Media

The mCCD cells were cultured in Dulbecco's Modified Eagles Media (DMEM) / F12 Media 1:1 (Gibco cat #31331) supplemented with 10 ml foetal calf serum (Invitrogen cat #10270-106), 5 ml Insulin Transferrin Selenium (ITS) solution (Gibco cat #41400-045), 5 ml Penicillin Streptomycin (Invitrogen cat #15140-122), 50 μl 500 nM Dexamethasone (Sigma Aldrich cat #D2915), 500 μl 1 μM T3 (Sigma Aldrich cat #T6397) and 500 μl 10 μg/ml EGF (Sigma Aldrich cat #E4127).

2.10.2. Cell passaging

The mCCD cells were grown to near confluency in tissue culture treated flasks prior to passaging. Cells were passaged by removing the media and twice incubating the cells with DPBS supplemented with 1 mM Ethylenediaminetetraacetic acid (EDTA) for 10 minutes. This solution was then replaced with 1ml of trypsin EDTA solution (Lonza cat #BE17-161E) for each 25 cm^2 of flask surface and the cells incubated at 37 °C for approximately 10 minutes or until the majority of the cells had detached from the flask surface. The resuspended cells were then diluted with a 6 fold excess of fresh media and then one quarter of the resuspended cells seeded onto fresh flasks.

2.10.3. Exosome depleted media

Purification of exosomes from the tissue culture conditioned media assumes that the exosomes present are released by the cells. Serum is a rich source of exosomes and other small lipid membrane bound vesicles which unless removed will interfere with the detection of exosome release from the cells. Unfortunately without foetal calf serum supplemented into the tissue culture media cell viability is compromised. Exosome depleted foetal calf serum was made by diluting the foetal calf serum with media to a concentration of 20% which reduced the viscosity of the solution facilitating the pelleting of the exosomes under centrifugation. The 20% foetal calf serum solution was centrifuged overnight at 200,000 x *g*. The supernatant was removed and transferred to fresh tubes before being centrifuged for a further hour at 200,000 x *g*. The supernatant was again removed and after being filtered through a 0.22 μm cellulose acetate filter could be used in tissue culture without interfering with the detection of exosomes released from the cells.

2.10.4. AnnexinV/PI apoptosis/necrosis determination

The Annexin-V-FLUOS staining kit (Roche, cat # 11 858 777 001) was used for quantification of apoptosis and necrosis. Firstly the cells were released from the flask surface by treatment with trypsin. Following the protocol for passaging, the cells were incubated twice with PBS supplemented with 1 mM EDTA at room temperature for 10 minutes. This was then replaced with trypsin solution and incubated at 37 °C. Once the cells had detached from the flask surface a 6 fold excess of media to trypsin was added to neutralise the trypsin and the cells pelleted at 500 g for 3 minutes. The cells were resuspended in PBS and then centrifuged again before resuspension in the incubation buffer supplied with the kit. The resuspended cells were then

split into two tubes. One tube was supplemented with the incubation buffer only and the second tube supplemented with incubation buffer plus Annexin-V-Fluorescein each to a final volume of 0.5 ml. After incubation at room temperature for 10 minutes each sample was analysed on a BD Biosciences FACS Calibur. Propidium iodide was then added to the Annexin-V-Fluorescein labelled sample and analysed immediately on the FACS Calibur. The Fluorescein response was measured on the FL1 channel and the propidium iodide response was measured on the FL3 channel.

2.10.5. Stimulation with ionomycin/vasopressin/cisplatin

Flasks of confluent cells were washed in foetal calf serum free media and then media containing exosome depleted foetal calf serum added. Ionomycin (Sigma Arich cat #I0634), desmopressin (dDAVP; Sigma Aldrich, cat #V1005), and cisplatin (Calbiochem, cat #232120) were diluted in serum free media and then added to the flasks at the necessary concentration. The mCCD cells were stimulated with ionomycin for 2 hours and cisplatin for 48 hours after which the media, and for the cisplatin treatment the cells, were collected. Stimulation with desmopressin lasted 96 hours. The media was collected at 48 hours and then replaced. Both the cells and the media were collected at 96 hours. For each stimulation collection of the cells was achieved by scraping them into ice cold RIPA solution (50 mM TRIS, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS; pH 7.4) after first washing the cells in DPBS. The cell suspension was then centrifuged for 5 minutes at 15,000 x g and the supernatant stored at -80 °C prior to analysis.

2.10.6. Effect of added exosomes

Cells were grown to confluency in two T150 flasks and then stimulated with 3.16 ng/ml dDAVP or left unstimulated for 96 hours. The media was changed at 48 hours. The media was collected at 96 hours and the exosomes purified. The protein concentration in the purified exosome samples was measured using the BCA assay. The mCCD cells were passaged and then grown to confluency in a 12-well plate. The purified exosomes were added to a well at a concentration of 50 μg/ml. Stimulation with 3.16ng/ml dDAVP was used as a control. After incubation for 48 hours the cells were washed with DPBS and then scraped into ice cold RIPA solution. The cell suspension was then centrifuged for 5 minutes at 15,000 x *g* and the supernatant stored at -80 °C prior to analysis.

2.11. Data Analysis

Data generated by FACS was analysed using the BD Biosciences CellQuest software package. The intensity of bands on western blots was measured using the BioRad Quantity One v4.5.0 software package. The DataAnalysis v1.3 software package from Bruker Daltonics was used for the analysis of the mass spectrometry data in chapter 3. Version 1.4 was used in chapters 5 and 6. The Progenesis LC-MS v2.6 software package from Non-linear Dynamics was used for the quantitative mass spectrometry in chapter 6 following pre-processing using the DataAnalysis software. The MASCOT server from Matrix Science [119] was used to identify proteins from the MS/MS data. Information on the identified proteins was gathered using the Harvester website [120] which includes the Online Mendelian Inheritance in Man (OMIM) website [121]. The Exocarta website was used to find previous instances of a protein being detected in exosomes [122]. Data analysis was performed in the python language with the Scipy [123] and Matplotlib [124] packages used for general statistical analysis and data visualisation respectively. Experimental measurements were summarised with mean and SEM. Statistical significance was accepted at $p<0.05$.

3. Human urine contains exosomes

3.1. Introduction

Urine is enriched for organ-specific proteins originating from the kidney and urinary tract [125] and is the logical biofluid to study to identify kidney disease biomarkers [103]. There are significant challenges to overcome if a study is to be successful. The urinary proteome is more dilute than the plasma (although this is somewhat mitigated by the ease of collecting urine in comparatively large volumes), analysis is often complicated by interfering compounds such as salts and, although less complex than the plasma proteome, it is still sufficiently complex to present a challenge [126]. The soluble urinary proteome includes proteins filtered from the plasma and proteins released directly by the kidneys [104]. Exosomes should be enriched for proteins released by the kidneys and present a partial solution to the complexity and large dynamic range of the urinary proteome [127].

Several groups have previously reported the presence of exosomes in urine [10, 40]. The first objective of this chapter was to establish a protocol for the purification of exosomes from human urine and confirm their presence using established criteria [1].

The second objective was to explore the proteome of the urinary exosomes using mass spectrometry and so expand the list of currently identified exosomal proteins.

The third objective was to investigate whether the exosomal proteome changes with disease. The usefulness of the exosomes as a source of biomarkers is dependent on the exosomal proteome changing in response to disease state.

The fourth objective was to investigate the ability of label-free quantitative mass spectrometry to accurately quantify changes in protein concentrations between samples. Label-free quantitative mass spectrometry has potential advantages over other methods of proteome-wide quantitation but is a relatively new technique and validation of the approach is needed before committing to a large scale study which depends on the technique.

3.2. Methods

This study was performed with local ethical approval (MREC number: 06/MRE00/67) and informed consent was obtained from all participants or their next of kin. Healthy, male volunteers (aged 27±3.27) were recruited from the Queen's Medical Research Institute, University of Edinburgh. Subjects with sepsis (Table 3.1) were recruited from the Intensive Therapy Unit, Edinburgh Royal Infirmary. Urine was collected in the morning using sterile 50 ml centrifuge tubes. The method used for the purification of exosomes is described in Chapter 2.1.1. Briefly, urine was centrifuged at 15,000 x *g* to pellet any cell debris and then the supernatant was centrifuged at 200,000 x *g* to pellet the exosomes. The presence of exosomes was verified using SDS-PAGE (Chapter 2.3.1.), western blot (Chapter 2.4.), isopycnic centrifugation (Chapter 2.5.) and transmission electron microscopy (Chapter 2.6.1.). The Bio-rad gel system was used for SDS-PAGE.

	Sepsis 1	**Sepsis 2**	**Sepsis and AKI 1**	**Sepsis and AKI 2**	**Sepsis and AKI 3**
Sex	F	F	F	M	M
Age	25	40	22	70	61
Temperature (°C)	36.5	38	>38	38	37.5
Mean arterial pressure (mmHg)	87	85	70	95	70
Heart rate (BPM)	115	90	130	105	120
Respiratory rate (min^{-1})	33	28	>20	35	26
pO_2 (kPa)	9.7	13.16	15.51	12.65	12.42
pCO_2 (kPa)	7.99	6.15	6.35	3.12	4.43
H^+ (nM)	58.7	37.5	48	23.5	38.1
FIO_2	0.95	0.55	0.5	0.4	0.28
Serum Sodium (mmol/L)	138	140	139	144	128
Serum Potassium (mmol/L)	4.5	4.6	4.1	3.7	4.9
Serum Creatinine (μmol/L)	59	84	355	258	237

	Sepsis 1	**Sepsis 2**	**Sepsis and AKI 1**	**Sepsis and AKI 2**	**Sepsis and AKI 3**
Serum Bilirubin (μmol/L)	18	3	10	11	39
Haemoglobin (g/L)	97	69	63	68	84
WCC ($x10^9$/L)	35.4	11.2	28	15.7	12.3
Platelets ($x10^9$/L)	38	389	441	633	232
Urine output in 24 hrs (mL)	1130	2256	1500	3855	3252

Table 3.1 Demographic and physiological information on the five patients with sepsis whose urine was used to study changes in the urinary exosomal proteome.

The proteome of the urinary exosomes was investigated using GeLC-MS/MS as described in Chapter 2.9.1. To reduce the complexity of the sample entering the mass spectrometer GeLC-MS/MS separates the proteome based on the size of the intact proteins using SDS-PAGE. In-gel tryptic digestion is performed on gel slices and the extracted peptides are then separated based on their hydrophobicity using HPLC prior to tandem mass spectrometry. The resulting data was analysed on the Bruker DataAnalysis software package. The MASCOT server version 2.2 from Matrix Science [128] was used to identify proteins from the MS/MS data. Mascot searches were performed on the NCBI (NCBI_Fi 20080229) database (6251073 sequences) restricted to *Homo sapiens* (199930 sequences) with the following parameters: a specified trypsin cleavage with a maximum of 1 possible missed cleavages, precursor and fragment ion tolerances of ±1.2 Da and ±0.6 Da respectively, fixed modification caused by carbamidomethylation on cysteines and variable modification by oxidation on methionine. Individual ion spectra were accepted at scores equivalent to $p < 0.05$.

Label-free quantitative mass spectrometry was performed on samples spiked with peptides following digestion and spiked with proteins prior to digestion. The complete protocol for each experiment is documented in chapter 2.7.4. Briefly, for the first experiment two aliquots of purified urinary exosomes digested with trypsin were spiked with 5 peptides to represent 1% and 4% of total protein by mass. The two aliquots were then analysed by LC-MS/MS. The peaks corresponding to the spiked peptides were then located in the spectra and the area of the peaks compared between the samples.

For the second experiment two aliquots of urinary exosomes were spiked with six proteins to represent 1% and 4% of total protein by mass. GeLC-MS/MS was performed on each aliquot. The peptides derived from the spiked proteins were identified by querying the MASCOT software. The peak area for each of the identified peptides was measured and then compared between the two aliquots.

3.3. Results

3.3.1. Exosome detection in human urine

To investigate whether exosomes were being purified from the urine in the ultracentrifuge pellet the components of the supernatant and pellet were separated by SDS-PAGE and probed for three markers of exosomes. TSG101 is a component of the ESCRT-I complex which is involved in the formation of the intralumenal vesicles which are eventually released as exosomes [1]. Flotillin 1 is thought to be present at the site of exosome formation and incorporated in their proteome [1]. CD24 is a GPI-linked mucin-like adhesion molecule demonstrated to be a marker of urinary exosomes [40]. TSG101, flotillin 1 and CD24 are all present in the ultracentrifuge pellet but are not detected in the supernatant (Figure 3.1).

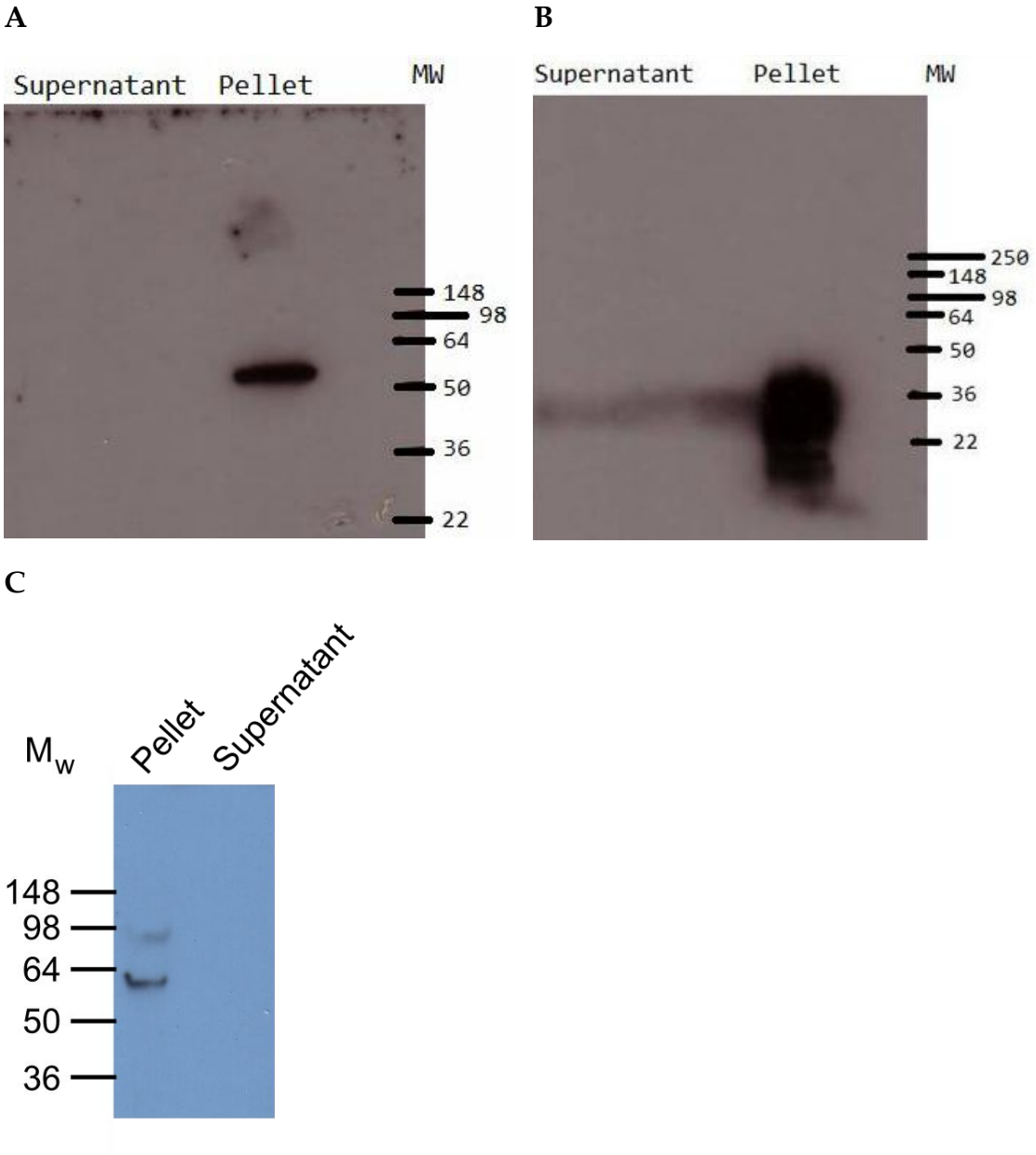


Figure 3.1 Western blot for exosomal markers.
In human urine the exosomal markers A) TSG101, B) CD24 and C) flotillin 1 are enriched in the ultracentrifuge pellet compared with the supernatant. Each panel is representative of three separate experiments.

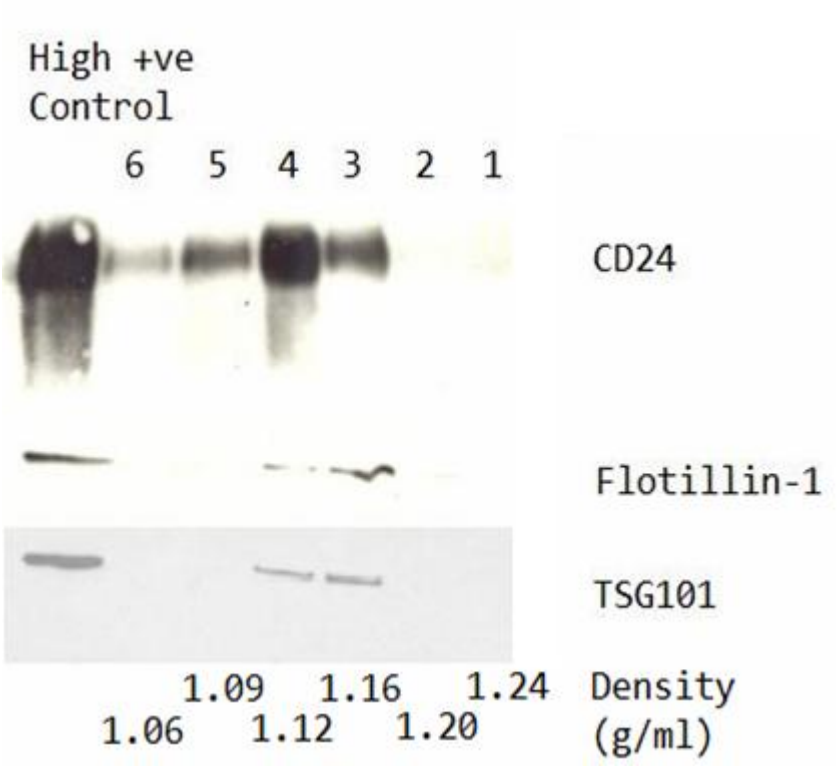


Figure 3.2 Western blot on isopycnic centrifugation fractions.
In human urine, the exosomal markers flotillin 1, TSG101 and CD24 localise to a density of 1.12-1.16 g.cm^{-3} on a sucrose density gradient following isopycnic centrifugation. This distribution was consistent across five biological replicates with the exosomal markers within the range 1.10-1.17 g.cm^{-3} for all.

The components of the ultracentrifuge pellet were separated by isopycnic centrifugation on a density gradient to investigate whether the markers are present at the characteristic exosomal density of 1.10-1.15 $g.cm^{-3}$ [116]. From the density gradient six fractions were collected with the exosomal markers localised in fractions 3 and 4 (Figure 3.2). This corresponded to a density of 1.12-1.16 $g.cm^{-3}$.

As a final step in verifying that exosomes are present in the ultracentrifuge pellet they were directly visualised by transmission electron microscopy. Several structures of the characteristic size of 20-100 nm and cup-shaped appearance [116] of exosomes were identified (Figure 3.3).

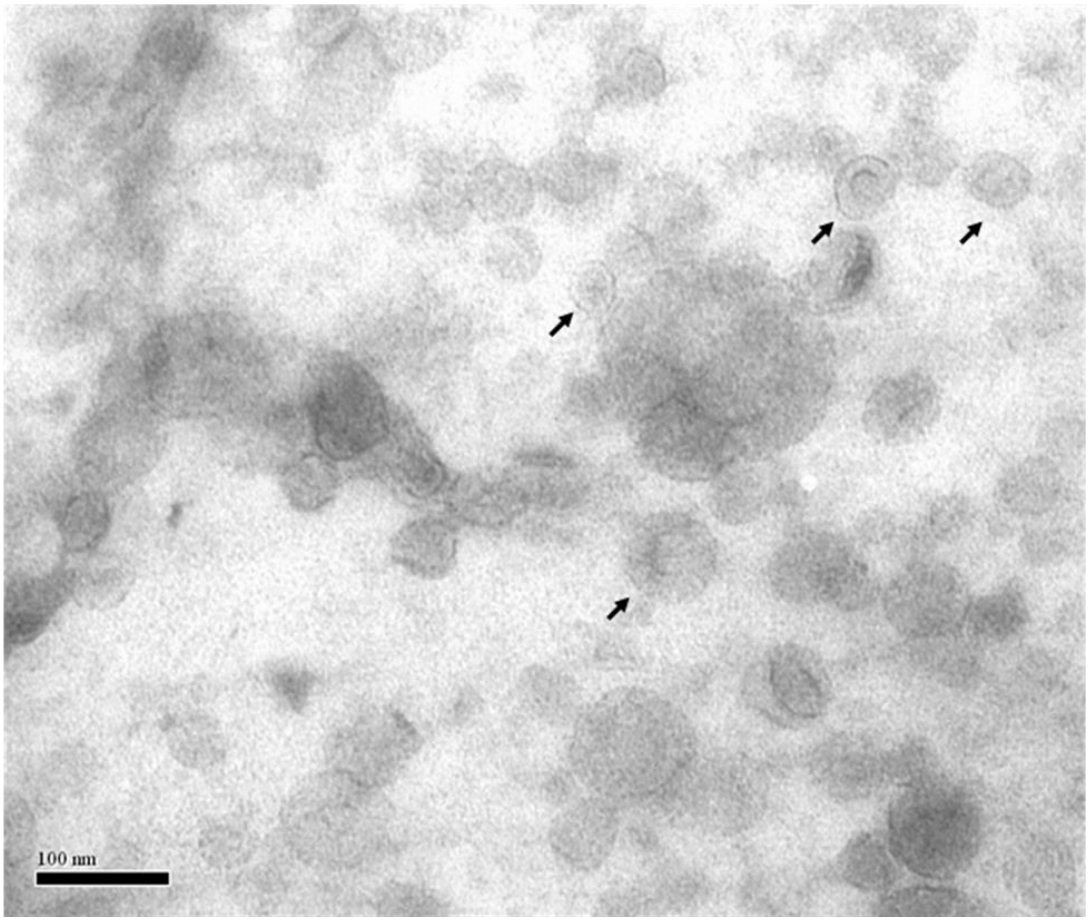


Figure 3.3 Transmission electron micrograph of embedded and negatively stained human urinary exosomes.
Exosomes have previously been described as 20-100 nm in diameter and cup-shaped in appearance. Several structures of the characteristic size and appearance of exosomes are highlighted by arrows.

3.3.2. Analysis of the urinary exosomal proteome

With the presence of exosomes established their proteome was investigated by GeLC-MS/MS. The urinary exosomes of a healthy volunteer were analysed. The gel was sliced into 10 sections during GeLC-MS/MS. The slices were not equally spaced through the gel. Instead slice boundaries were chosen to limit the presence of the high abundance proteins to the smallest sections of gel possible (Figure 3.4). Using this technique 76 proteins were identified (Table 3.2 and Appendix 1). Further information on these identified proteins was collected from the harvester website [120]. 42% of the identified proteins were cytoplasmic in origin, 30% were either integral or linked to the plasma membrane, 15% were from other sections of the cell and 13% were extracellular in origin (Figure 3.5). A small number of the identified proteins have previously been shown to be specific to various sections of the kidneys (Figure 3.6). The identified proteins were queried in the Online Mendelian Inheritance in Man (OMIM) database [121] which revealed that 9 of the identified proteins were linked to renal disease and a further 3 were linked to endosomal diseases (Table 3.3).

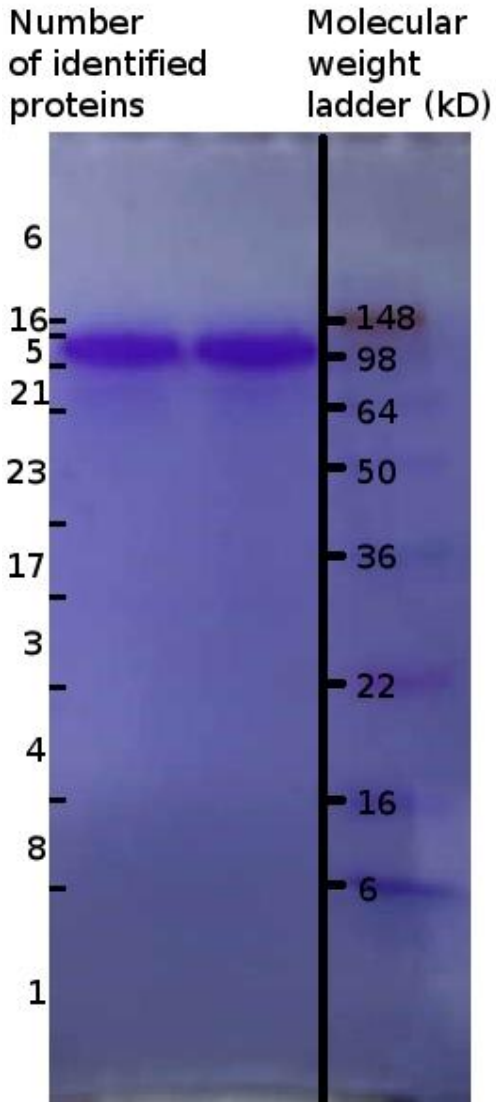


Figure 3.4 A human urinary exosomal protein sample run on a 1D SDS-PAGE gel and stained with colloidal brilliant blue.
Urinary exosomes from one individual were used. The gel was sliced into 10 sections, shown along the left edge. The proteins in each section were then digested with trypsin and the peptides identified using mass spectrometry. The number of proteins identified in each section is shown along the left edge of the gel. The markers present in the ladder are labelled on the right.

Protein	Accession ID	Subcellular location	Maximum ions per band	Mascot Score	Previously identified in exosomes
TSG101(Uev) Domain In Complex With Ubiquitin	1S1Q_B	Cytoplasm (endosomal trafficking)	3	266	[10, 129-131]
Alix	2OEX_A		2	73	[130, 132-134]
Annexin A11	NP_001148		3	73	[10, 130-132]
Annexin A2 isoform 2	NP_004030		2	59	[132]
Annexin A4	AAC41689		2	46	[10, 130-132, 134]
RAB10, member RAS oncogene family	NP_057885		3	45	[10, 130-132]
Ubiquitin and ribosomal protein L40 precursor	NP_003324	Cytoplasm (soluble)	3	266	
Polyubiquitin	BAA23486		2	266	
Lactate dehydrogenase B	NP_002291		4	157	[10, 130-133, 135]
Olfactomedin 4	AAH47740		3	153	[10, 131, 134]
Glyceraldehyde-3-phosphate dehydrogenase	CAA25833		3	147	[131]
FBP aldolase I a	BAF83484		3	137	[10, 131, 132, 134-136]
S100 calcium-binding protein A9	NP_002956		2	131	[131, 134]
BROX	NP_653296		3	116	[10, 130-132, 134]
Carbonic anhydrase II	NP_000058		1	93	[10, 131]
Tyrosine 3/tryptophan 5 -monooxygenase activation protein, epsilon polypeptide	NP_006752		1	93	[10, 130-134]
Protein expressed in prostate, ovary, testis, and placenta 2	NP_001077007		3	93	[132]
2-phosphopyruvate-hydratase alpha-enolase;	CAA59331		2	89	

carbonate dehydratase					
Enolase 3	CAA34513		2	79	[10, 130]
Lactate dehydrogenase A	BAC85389		2	76	[10, 29, 130-132]
Calbindin D28K	AAB19408		1	76	[10, 131]
Peptidylprolyl isomerase A (cyclophilin A)	NP_066953		2	74	[10, 129-132, 134, 135]
c-myc binding protein	EAX07292		2	64	
G protein beta subunit	AAA35922		2	60	
S100 calcium binding protein A8	CAA68390		2	59	[131, 134]
Alpha globin	CAA23749		1	55	
Phosphoglycerate kinase 2	CAA28872		1	49	
Phosphoglycerate kinase 1	NP_000282		2	49	[10, 29, 130, 132, 134]
Membrane alanine aminopeptidase precursor	BAD93155	Plasma membrane (integral)	26	962	[10, 131, 134]
gp330 precursor	AAB41649		16	574	[10, 131]
Prominin 1	NP_006008		5	290	[10, 129, 131, 134]
MME Neprilysin	CAA30157		5	182	
G protein-coupled receptor (GPRC5C)	CAC00633		4	145	[10, 129, 131]
Gamma-glutamyltransferase 1	AAA52546		1	122	[10]
Bile salt export pump	AAD28285		1	120	
Maltase-glucoamylase	NP_004659		3	119	[10]
NaCl electroneutral Thiazide-sensitive cotransporter	CAA62613		5	111	
Mucin-1	CAA36478		2	111	[10, 129, 131, 134, 135, 137]
Orphan G-protein coupled receptor	AAF67321		1	66	[10, 131, 134, 135]

(GPRC5B)					
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	EAW94315		3	94	[10, 130, 131]
Fructose transporter	AAB60641		2	80	
Napsin A preproprotein	NP_004842		1	80	
Aquaporin 1	AAC16481		1	77	[10, 131]
Desmoglein-1 precursor	Q02413		1	76	
Solute carrier family 12 member 1 (Bumetanide-sensitive sodium-(potassium)-chloride cotransporter 2)	Q13621		4	55	[10, 131]
CD26	CAA43118		1	48	[10, 29, 44, 131, 132, 134]
Tetraspan TM4SF (Tspan-1)	AAC69714		1	45	[10, 129-131]
Galectin 3 binding protein	NP_005558	Extracellular (secreted)	6	679	
Mannan-binding lectin-associated serine protease-2 (MASP-2)	CAA67050		2	389	[10, 131, 138]
Pro-epidermal growth factor precursor (EGF)	P01133		5	223	[10, 131]
Serum Albumin	2I2Z_A		10	222	[10, 131, 132, 134, 138, 139]
Beta-galactosidase related protein precursor	AAA35599		5	191	
ACPP acid phosphatase	CAA36422		2	106	[10, 131]
Saposin A	2DOB_A		2	88	
Apolipoprotein J precursor	AAA51765		1	60	
Plasma serine protease inhibitor precursor	AAA35688		3	58	
Cubilin	AAC82612	Plasma membrane (peripheral)	7	338	[10, 131]
Syntenin	AAB51246		2	125	[10, 130-135]

Aminopeptidase A	AAA35522		6	122	[10, 131]
Ezrin	CAA35893		2	117	[10, 129, 132, 133, 135, 140]
CD59 antigen p18-20	NP_000602		1	50	
Beta actin variant	BAD96645	Cytoplasm (cytoskeletal)	6	149	[10, 29, 44, 129, 130, 132-135, 137, 139-141]
Alpha-actin	AAA51577		3	68	[10, 130, 132]
Putative heart protein (PHP)	AAC73009	Lysozyme	2	114	
Cerebroside sulfate activator protein	AAA36594		2	105	
Lysosomal pepstatin insensitive protease	AAB80725		1	101	
Cathepsin A	EAW75788		2	93	[10, 131]
Uromodulin	AAH35975	Plasma membrane (GPI linked)	73	4930	[10, 131]
Dipeptidase 1 (renal)	NP_004404		5	199	[10, 29, 130, 131]
Histone cluster 1, H2bl	NP_003510	Nucleus	2	63	
Putative MAPK-activating protein PM28	P53990	ER/Golgi	3	190	
Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	NP_006487		2	46	[10, 130-132]
Podocalyxin-like	BAB71022	Unclassified	3	106	[10, 131]
Beta-trace 23.5 kda glycoprotein	AAB24507		1	46	
Apolipoprotein D (apoD)	AAB32200		2	61	[10, 131, 138]

Table 3.2 All proteins identified by GeLC-MS/MS in the urinary exosomal proteome of a single individual.
Protein identifications from the GeLC-MS/MS data were made using the MASCOT service and the NCBI database using a $p<0.05$ cut-off. The subcellular location for each protein was discovered by querying the harvester website

(http://harvester.fzk.de/harvester/). Instances of a protein previously being identified in exosomes are based on references from the exocarta website (http:// exocarta.ludwig.edu.au/).

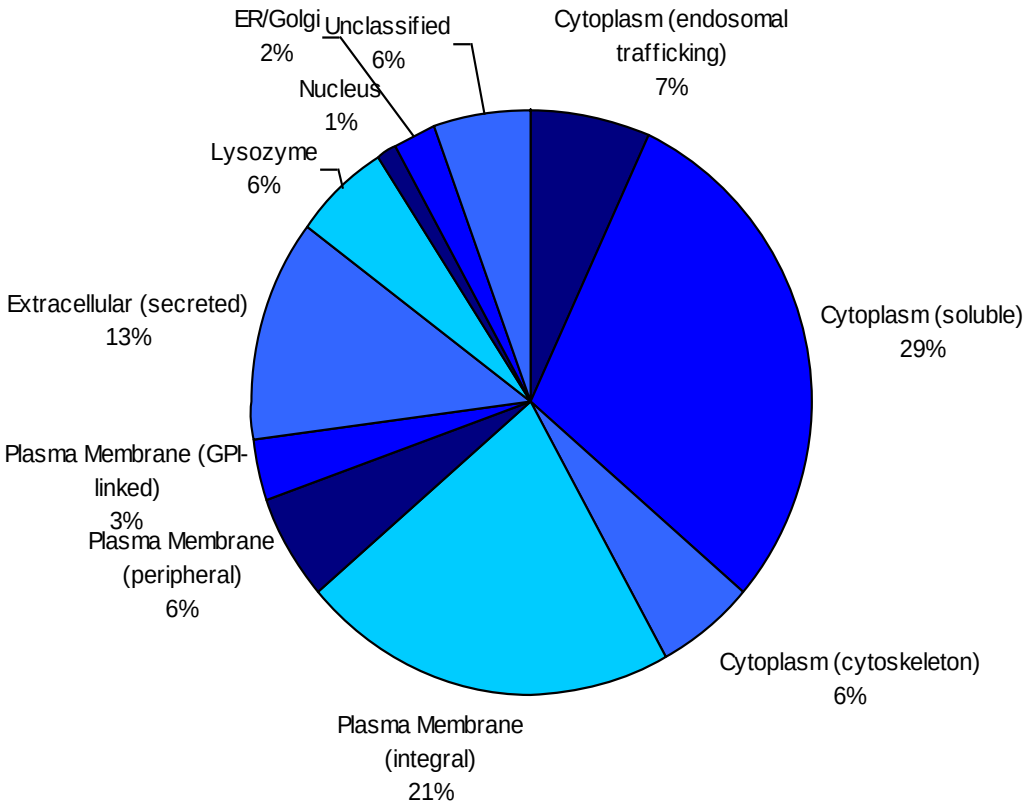


Figure 3.5 Sub-cellular location of proteins identified by GeLC-MS/MS. In the human urinary exosomes obtained from one individual 76 proteins were identified by mass spectrometry (Table 3.2). These proteins were classified based on their sub-cellular location following an analysis using the Harvester website.

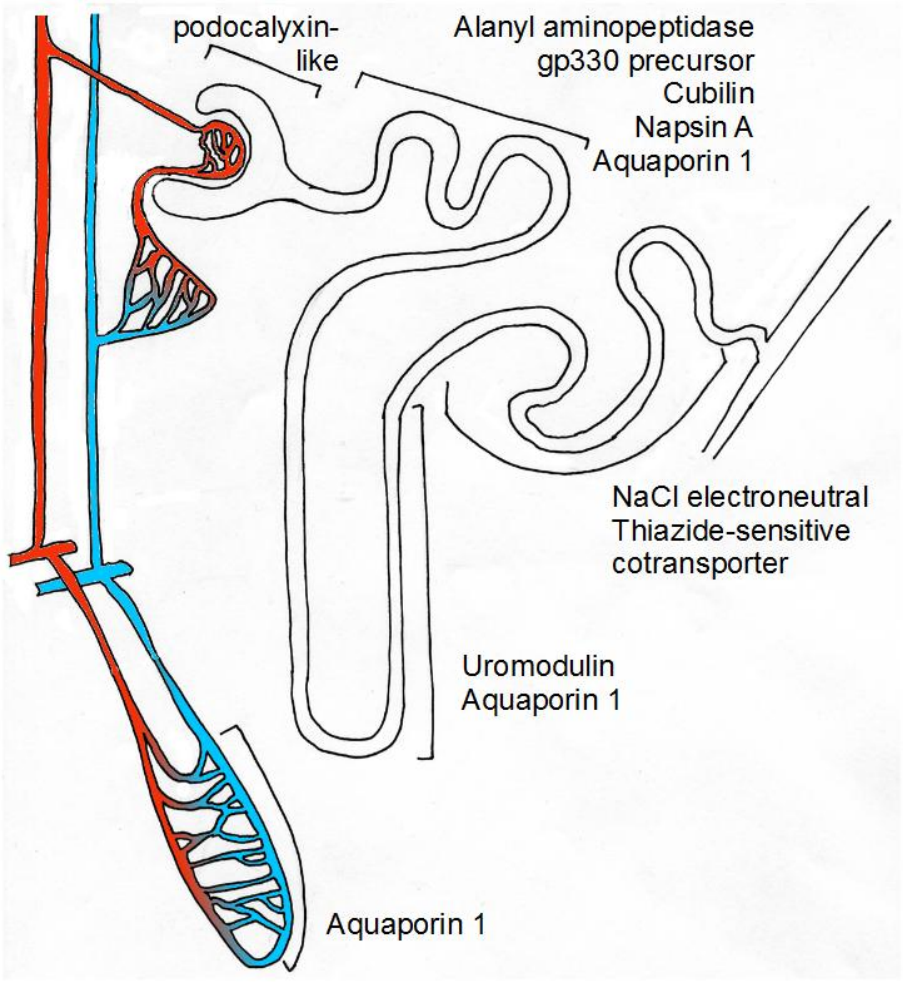


Figure 3.6 Proteins specific to nephron sections.
The proteins identified in the urinary exosomes of one individual (Table 3.2) were analysed using the Harvester website. Several proteins were found to be specific to or enriched in regions of the kidney.

Protein	Disease associated
Renal Diseases	
Uromodulin	Hyperuricemic nephropathy Medullary cystic disease 2 [142]
gp330 precursor	renal aminoglycoside accumulation and nephrotoxicity [143] Donnai-Barrow syndrome [144]
Pro-epidermal growth factor precursor (EGF)	normocalciuric renal hypomagnesemia [145]
MME Neprilysin	membranous glomerulonephritis [146]
Putative heart protein (PHP)	Farber disease [147, 148]
NaCl electroneutral Thiazide-sensitive cotransporter	Gitelman syndrome [149]
carbonic anhydrase II	osteopetrosis and renal tubular acidosis [150]
angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	renal tubule dysgenesis [151], diabetic nephropathy [152]
Solute carrier family 12 member 1 (Bumetanide-sensitive sodium-(potassium)-chloride cotransporter 2)	Bartter syndrome [153]
Endosomal Diseases	
Beta-galactosidase precursor	GM1-gangliosidosis [154]
Cerebroside sulfate activator protein	Metachromatic Leukodystrophy [155], Atypical Gaucher Disease [156]
Protective protein for beta-galactosidase	galactosialidosis

Table 3.3 Urinary exosomal proteins identified by GeLC-MS/MS which are associated with disease.

The proteins identified in the urinary exosomes of one individual (Table 3.2) were analysed using the OMIM website for any associations with disease.

3.3.3. Label-free quantitative mass spectrometry

With the presence of exosomes in the urine established the opportunity exists to explore their proteome as a potential source of biomarkers for disease. This opportunity is based on the hypothesis that the proteome of the urinary exosomes changes with disease. As a preliminary step in verifying this hypothesis the urinary exosomes of patients with sepsis were compared with the urinary exosomes of healthy controls using SDS-PAGE and western blot. Using this approach cJun was identified as present in the urinary exosomes of all 5 septic patients studied but absent, or below the limit of detection, in the urinary exosomes of all 3 healthy controls studied (Figure 3.7).

Biomarker discovery requires the comparison of samples from healthy controls and patients with the illness being investigated. Label-free quantitative mass spectrometry is a promising new approach for quantitative comparisons. To investigate its suitability for studying the exosomal proteome two experiments using spiked samples were devised. The first experiment used peptide spikes to investigate the accuracy of just the mass spectrometry and data analysis steps. The second experiment used protein spikes to also investigate the reproducibility of the protein digestion.

The ratios for 4 of the 5 spiked peptides compared well with the expected values (Figure 3.8). The fifth peptide was poorly detected and the measured fold change deviated further from the expected value. For the spiked proteins 4 of the 6 proteins were identified by querying the mass spectrometry results against the MASCOT system [119]. The fold changes for individual peptides were more variable than for the earlier peptide spiking experiment. Much of this variability was removed by averaging the

fold changes across peptides for a protein although the experimentally measured fold change remained lower than the expected value for all four proteins (Figure 3.9.).

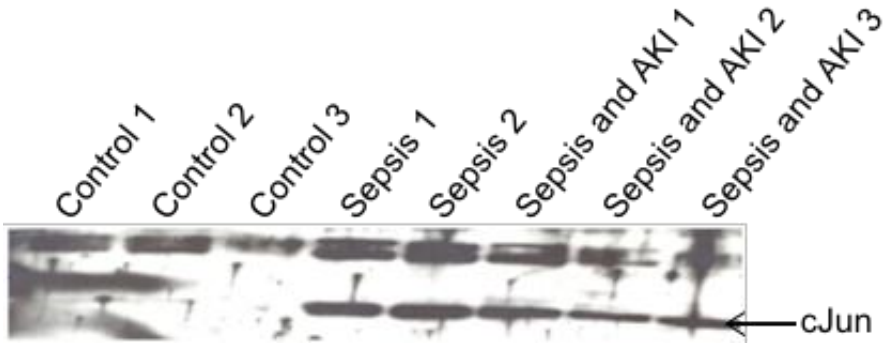


Figure 3.7 Western blot for cJun on urinary exosomes from healthy controls and septic patients.
cJun is increased in the urinary exosomes of septic patients.

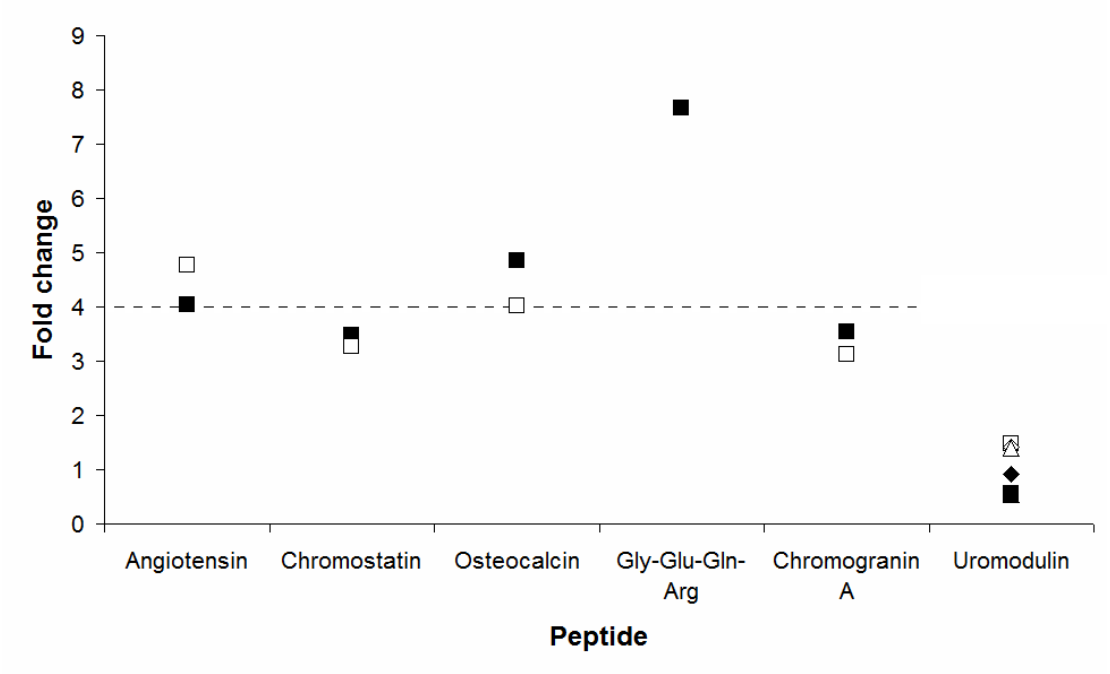


Figure 3.8 Fold change in spiked peptide samples.
Five peptides were spiked into trypsin digested urinary exosome samples at two different concentrations with one being four-fold higher than the other. The ion intensities for each of these peptides were then measured using the Bruker DataAnalysis software package. For four of the five peptides two charge states could be identified and each is shown by a separate point on the graph. For comparison the fold change in the ion intensities for several peptides derived from the uromodulin protein which were not spiked are shown.

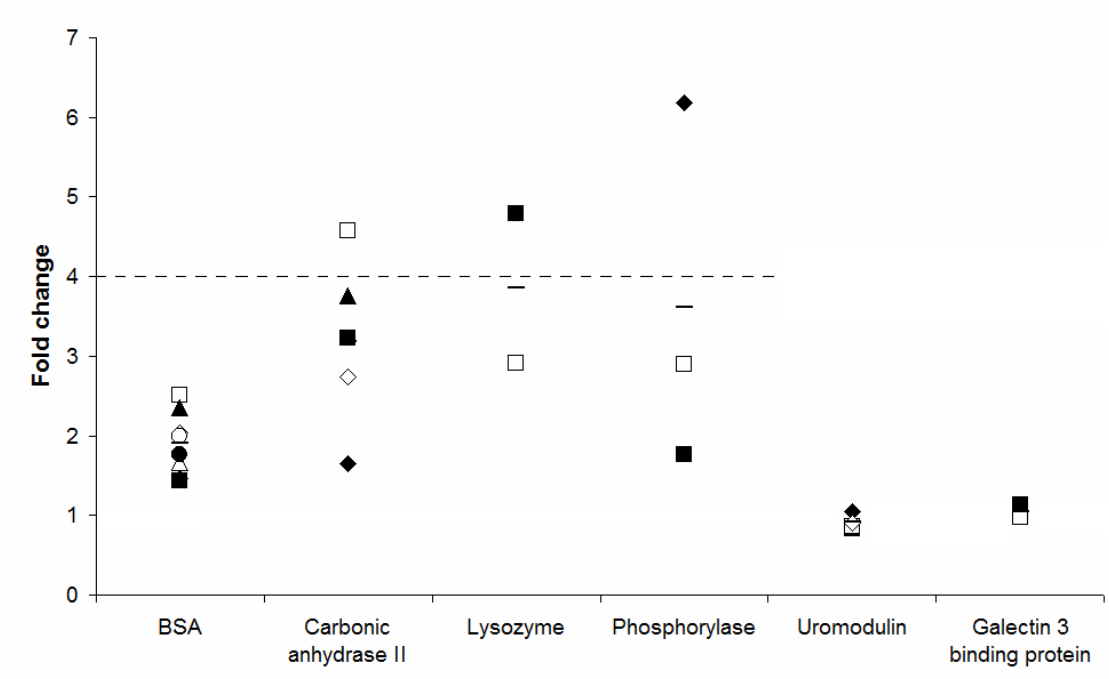


Figure 3.9 Fold change in spiked protein samples.
Six proteins were spiked into urinary exosome samples at two different concentrations with one being four-fold higher than the other. These samples were digested with trypsin and the resulting peptides analysed by LC-MS/MS. The peptides originating from the spiked proteins were identified using the MASCOT software for four of the proteins (BSA, carbonic anhydrase II, lysozyme and phosphorylase) and their ion intensities in each sample measured. For comparison the ion intensities for peptides derived from two proteins (uromodulin and galectin 3 binding protein) which were not spiked are also shown. Each point on the graph represents a single identified peptide and the horizontal bar represents the mean between peptides.

3.4. Discussion

Exosomes have previously been purified from the urine by several groups using ultracentrifugation [10, 40]. The protocol used varies between studies, perhaps to match the equipment available [116]. Based on the protocols of other groups and using the equipment available in our laboratory we have established a protocol for the purification of exosomes. Using western blot the exosomal markers TSG101, flotillin 1 and CD24 have been shown to be enriched in the ultracentrifuge pellet. Using isopycnic centrifugation we have established that these three markers are present on particles with a density of 1.12-1.16 $g.cm^{-3}$ which matches very closely with previous reports for exosomes [116]. Finally, using transmission electron microscopy we have directly visualised the particles in the ultracentrifuge pellet. Previous reports have described exosomes as cup-shaped in appearance and 20-100 nm in diameter [116] with more recent reports suggesting exosomes are predominantly at the lower end of this range [10, 40]. We were able to identify structures fitting this description. Taken with the other results the direct visualisation of exosomes means we can be confident in the isolation protocol. With the protocol established it should be directly transferable to other biofluids.

Using GeLC-MS/MS 76 proteins were identified from the urinary exosomal proteome. Only 13% of the identified proteins were known to be extracellular. Contamination of the exosomal preparation with protein from the soluble proteome is a potential concern but the high percentage of plasma membrane and cytoplasmic proteins, 30% and 42% respectively, in comparison to the much lower number of the extracellular proteins suggests this is not a significant problem. Searching the OMIM database for the 76

identified proteins revealed 12 which were linked to disease. Furthermore several of the proteins were known to be specific or enriched in certain sections of the nephron (for example, the presence of aquaporin 2 is consistent with exosome release from the collecting duct) suggesting that the exosomal preparation included exosomes which had been released from these regions of the nephron. These observations taken together suggest that the urinary exosomes represent a rich source of information, including biomarkers, on the entire length of the nephron, from the glomerulus through to the collecting duct.

Our interest in the exosomes as a potential source of biomarkers is based on the hypothesis that the proteome of the exosomes will change in response to the pathophysiological state of their cells of origin. Discovery-based proteomics should enable an unbiased search of the exosomal proteome for proteins which change between conditions but the technique is time consuming and costly. As a preliminary step a hypothesis-driven approach was used to identify changes in proteins we predict might change with sepsis. Transcription factors have previously been identified as potential biomarker candidates in exosomes [157]. Sepsis is an inflammatory condition so we investigated the inflammatory transcription factor cJun. In patients with sepsis we were able to detect cJun in their urinary exosomes but we could not detect cJun in the urinary exosomes purified from the urine of healthy controls. It is necessary to note several limitations with this small experiment. Firstly, the number of controls and patients was small, three healthy controls and five patients. Secondly, the two groups are not well matched. The healthy controls are considerably younger and are not hospital

patients. Despite these limitations this result suggests that a large scale discovery driven shotgun proteomics study should yield results.

The GeLC-MS/MS experiment could have been significantly expanded, by further fractionation of the gel and repeating the tandem mass spectrometry analysis, and yet despite the small scale still provided a wealth of information. Although improvements could be made to the existing protocol the search for biomarkers would be enhanced by quantitative data. A number of approaches for quantitative mass spectrometry have been developed utilising labels [158] but these involve adding processing steps which slow down the analysis, increase the cost and increase the errors. We decided instead to use a label-free approach because this potentially avoids many of the problems. Label-free quantitative mass spectrometry has only relatively recently become viable with improvements in the mass spectrometers and data analysis and as such the limitations of the technique are not as well understood [65, 159]. For this reason we began by investigating the accuracy of label-free quantitative mass spectrometry on spiked samples. Using peptide spikes allows the analysis of just the mass spectrometry and subsequent data analysis and in this experiment these steps did perform well. For four of the five peptides two ions could be detected and the experimentally measured fold change agreed closely with the expected value. The fifth peptide was poorly detected and the one ion which was detected deviated further from the expected fold change. Using protein spikes the accuracy of the entire experiment could be investigated. There was greater variability as would be expected with extra processing steps. Reducing the variability will be desirable but even with the level of variability seen in this experiment label-free quantitative mass spectrometry

could have potential as an initial screening tool. For all four proteins the fold change was underreported. This could potentially be due to reduced digestion and/or recovery of peptides from the sample spiked with the larger amount of protein. Switching from in-gel to in-solution digestion should reduce this effect and also reduce the variability.

Using western blotting for exosomal markers, density measurement by isopycnic centrifugation and direct visualisation by TEM we have confirmed that human urine contains exosomes and have demonstrated that our isolation protocol effectively isolates exosomes from the urine. Using GeLC-MS/MS we have begun to characterise the urinary proteome. We have demonstrated that the presence of cJun within the exosomes changes with sepsis and have evaluated the ability of label-free mass spectrometry to measure proteome-wide changes in the urinary exosomes.

4. Characterisation of the cortical collecting duct cell exosome in culture

4.1. Introduction

In chapter three techniques were developed to confirm the presence of exosomes in human urine [10, 40, 131]. These exosomes contain aquaporin (AQP) 2 indicating that, in vivo, exosomes are released from the collecting duct [10, 40, 131].

There is some evidence that the proteome of the kidney-derived exosome changes with cell stimulation or injury. For example, Zhou et al have demonstrated a change in the urinary exosomal proteome with disease. In a rat model of cisplatin induced acute kidney injury the exosomal protein fetuin A was demonstrated to increase with injury [160]. Although evidence is accumulating that the proteome does change with stimuli we do not fully understand the biological significance of such changes. Our understanding of the role of exosomes within the immune system is significantly more advanced, with a number of studies demonstrating that exosomes can transfer information between cells [1, 2, 19]. Study of cell culture models relevant to the kidney should increase our understanding of how the protein composition of the exosome changes with cell stimulation and allow us to determine if exosomes can transfer information between cells.

The kidney is a heterogeneous organ comprising different cell types each of which may respond differently to stimulation and the presence of exosomes. In choosing a cell line to study two competing factors were considered. Firstly, the estimated contribution of the nephron section to the pool of exosomes in human urine and, secondly, the exposure of the nephron section to exosomes in vivo. An established and characterised murine cortical collecting duct (mCCD) cell line [118, 161] was chosen because human

urinary exosomes have been repeatedly demonstrated to contain collecting duct proteins and, as the collecting duct is 'downstream' in terms of flow through the nephron, it is potentially exposed to exosomes from a range of cell types.

Our aim was to establish the mCCD cell line as a model for the study of exosomes. This model would be used to study the release of exosomes. The effect of exosomes on this cell line could then also be investigated.

4.2. Methods

The mCCD cells were raised from frozen stocks maintained within the laboratory following the generous gift of the cell line by Hans-Peter Gaeggeler, University of Lausanne, Switzerland [118]. The cells were routinely passaged using the protocol described in Chapter 2.10.2. The standard media (Chapter 2.10.1.) for this cell line includes 2% foetal calf serum (FCS). In early experiments it was demonstrated that the FCS contained exosomes which interfered with the detection of exosome release from the cells. To overcome this, exosome-depleted media was prepared by ultracentrifugation and filtration of 20% FCS as described in Chapter 2.10.3. and based on previously published protocols [116].

The exosomes released from the cells were purified from the media using the protocol previously established for the urine (Chapter 2.1.2 and 2.2.). The presence of exosomes was confirmed using SDS-PAGE (Chapter 2.3.1.), western blot (Chapter 2.4.), isopycnic centrifugation (Chapter 2.5.) and transmission electron microscopy (TEM; chapter 2.6.1.). The Thermo gel system was used for the SDS-PAGE.

The cells were stimulated with ionomycin, cisplatin and desmopressin (dDAVP) and as described in Chapter 2.10.5. Using FACS the effect of cisplatin and dDAVP on cellular apoptosis and necrosis was investigated. The protocol for FACS is outlined in Chapter 2.10.4.

The biological role of exosomes was investigated by collecting exosomes from mCCD cells stimulated with 3.16 ng/ml dDAVP for 96 hours and then applying them back on to unstimulated mCCD cells as described in Chapter 2.10.6.

4.3. Results

4.3.1. Exosome detection in mCCD cell conditioned media

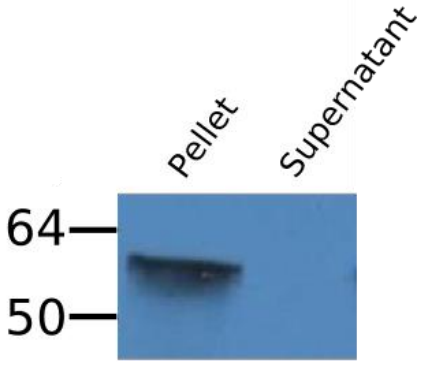
Ultracentrifugation of mCCD cell conditioned media forms a pellet which suggests that the mCCD cells release low density membrane vesicles. To establish whether the low density membrane vesicles released by the mCCD cells are exosomes the presence of two exosomal markers in the pellet was investigated. Ultracentrifugation of 20 ml of media obtained following a 48 hour incubation with a confluent T75 tissue culture flask yielded sufficient material for one or two separations by SDS-PAGE and subsequent western blot.

Flotillin 1 is thought to be present at the site of exosome formation and is often present in exosomes [1]. TSG101 is a component of the ESCRT-I complex and is directly involved in exosome formation. Flotillin 1 and TSG101 were both present in the ultracentrifuge pellet but not the supernatant (Figure 4.1).

The density of the particles containing flotillin 1 and TSG101 was determined using isopycnic centrifugation on a sucrose step gradient. Six fractions were collected from the gradient (Figure 4.2). The exosomal markers flotillin 1 and TSG101 together with AQP2 were predominantly localised to fractions 3 and 4 which corresponded to a density of 1.10 - 1.15 g.cm-3.

The particles in the ultracentrifugation pellet were directly visualised using TEM. Exosomes have previously been shown to vary in size from 20-100 nm and have a cup-shaped appearance. Structures matching this description in size and appearance could be easily identified (Figure 4.3).

A



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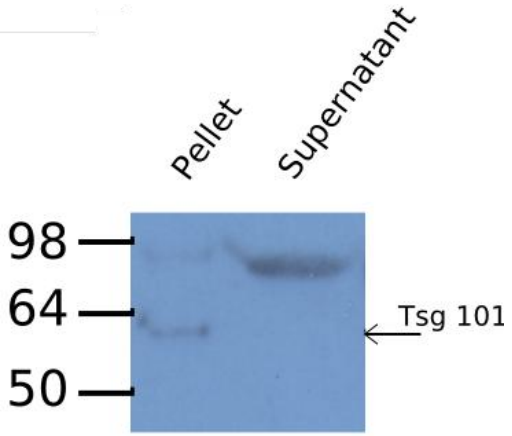
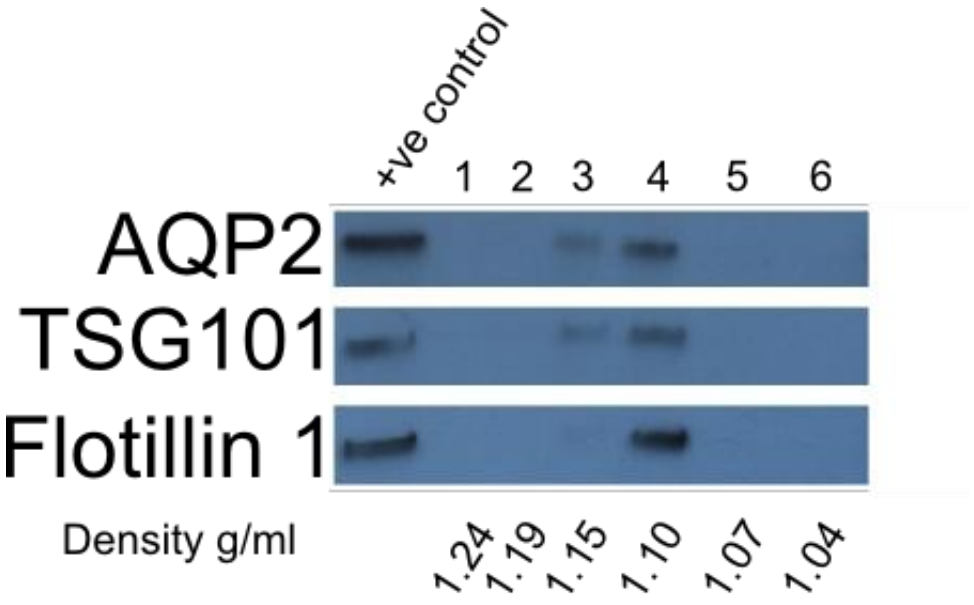


Figure 4.1 Western blot for exosomal markers on the ultracentrifugation pellet and supernatant from cultured collecting duct cells.
Both the exosomal markers A) flotillin 1 and B) TSG101 are enriched in the pellet. Each panel is representative of three separate experiments.

A



B

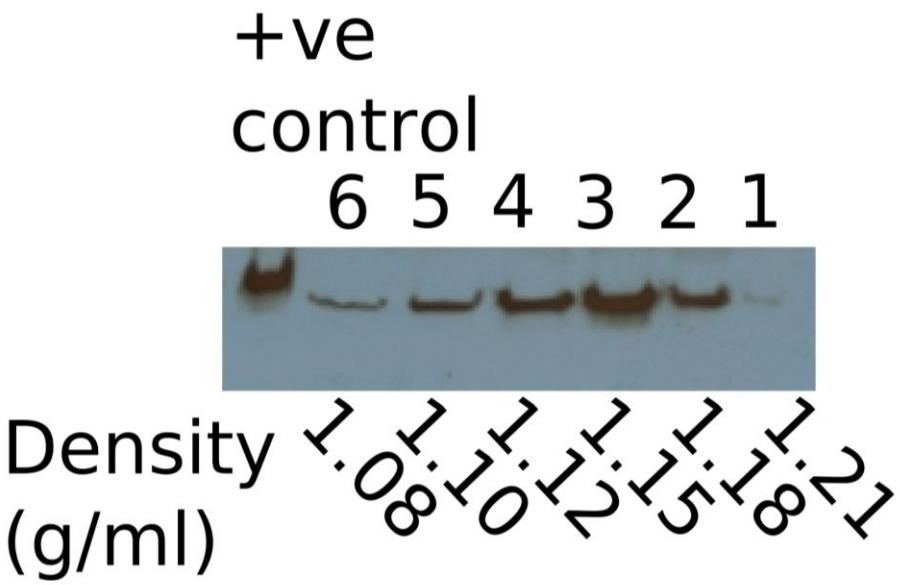


Figure 4.2 Western blot for AQP2, TSG101 and flotillin 1 on density fractions obtained following isopycnic centrifugation of dDAVP and ionomycin stimulated cell conditioned media.
A) Following stimulation with dDAVP AQP2, TSG101 and flotillin 1 are all present on particles with a density of 1.10-1.15 $g.cm^{-3}$ which is consistent with previous reports for exosomes. This distribution has been consistently observed over five replicates for TSG101 and flotillin 1 and three replicates for AQP2. The exosomal markers are present in the range 1.10-1.18 $g.cm^{-3}$ for all repeats. B) Flotillin 1, although concentrated at a density of 1.15 $g.cm^{-3}$ is present throughout the gradient following isopycnic centrifugation of exosomes derived from ionomycin stimulated cells.

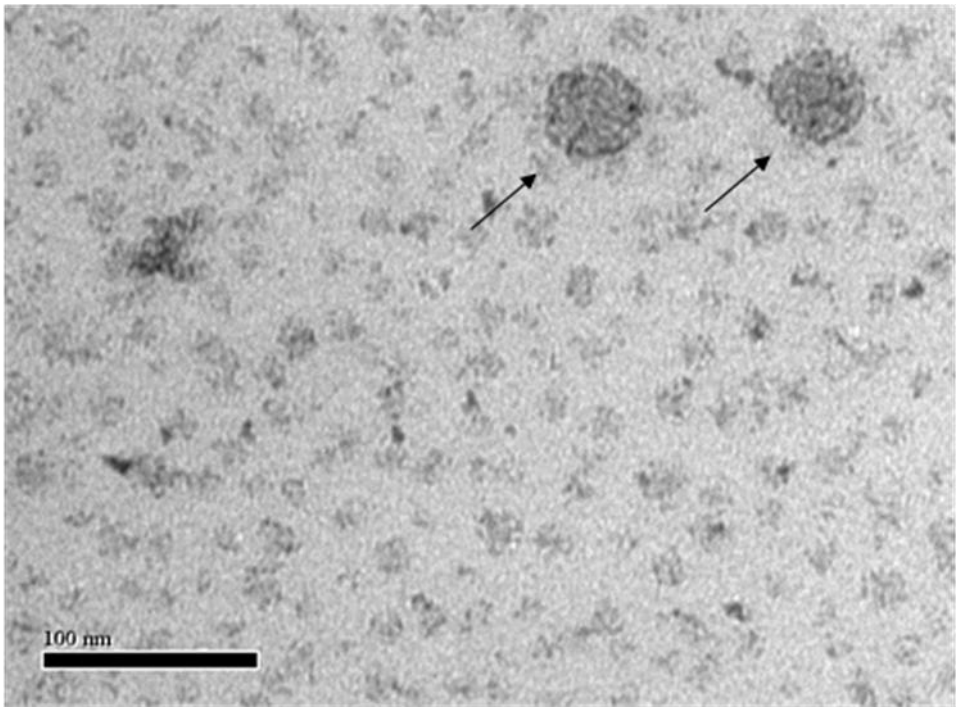


Figure 4.3 Transmission electron micrograph of exosomes.
The resuspended ultracentrifuge pellet was incubated and fixed on a formvar coated gold mesh grid which was then negatively stained with uranyl acetate and embedded with methyl cellulose. Under these conditions exosomes have been found to be 20-100 nm in diameter and have a cup-shaped appearance. Arrows highlight two such structures.

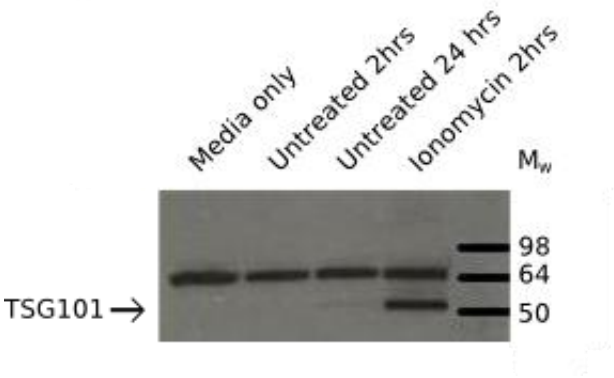


Figure 4.4 Western blot for the exosomal marker TSG101 on unconditioned media and unstimulated and ionomycin stimulated mCCD cell conditioned media.
TSG101 is increased with ionomycin treatment indicating exosome release.

4.3.2. Exosome release in response to stimuli

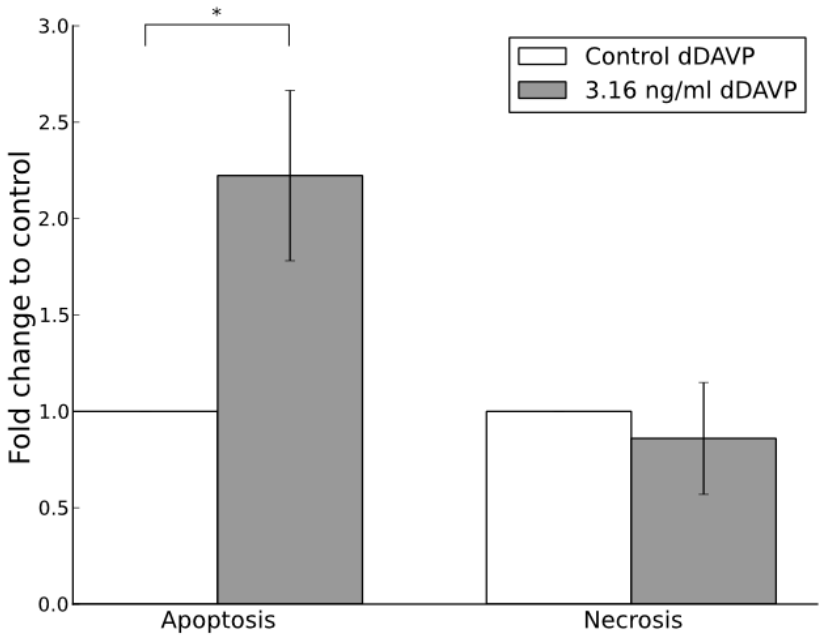
Release of exosomes has been shown to vary in response to a variety of different stimuli. Ionomycin is a calcium ionophore that facilitates the influx of calcium into the cell. Two hours after incubation with ionomycin there was more TSG101 present in the ultracentrifuge pellet than following 24 hours incubation without ionomycin (Figure 4.4).

Ionomycin is an artificial stimulus which the mCCD cells would not encounter under physiological conditions. Two factors were identified as models of physiological and toxicological insults. Desmopressin (dDAVP), a synthetic vasopressin analog, was chosen as the physiological stimuli. Stimulation with dDAVP should cause the upregulation of AQP2 in the cell line [161]. Cisplatin was chosen as the toxicological insult. Exosomal fetuin A has previously been identified as a potential biomarker in a rat model of cisplatin-induced acute kidney injury [160].

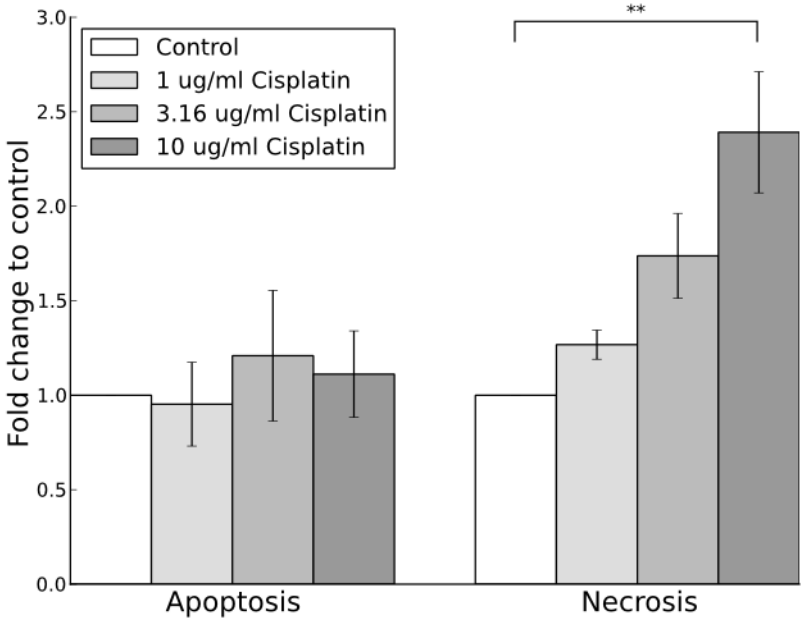
If the cells become apoptotic or necrotic they may release non-exosomal particles which could interfere with our study [116]. For example the calcium ionophore ionomycin induces a increase in the exosomal marker TSG101 in the ultracentrifuge pellet but the particles present spread throughout a gradient during isopycnic centrifugation (Figure 4.2B) indicating the presence of particles other than exosomes. To avoid this a concentration for dDAVP and cisplatin was determined which would not cause significant apoptosis or necrosis. In previous studies mCCD cells had been stimulated with dDAVP at a concentration of 1 ng/ml [161] without detrimental effect. Stimulation with 3.16 ng/ml dDAVP had no effect on cell necrosis but increased the proportion of apoptotic cells (Figure 4.5A;

p=0.014). However, the percentage of apoptotic cells was still less than 1% in all experiments. Stimulation with cisplatin at a concentration of 10 μg/ml caused a more than two-fold increase in the proportion of necrotic cells (8.2±0.8% compared to 3.6±0.5%, p=0.008) but no change in the proportion of apoptotic cells (2.3±1.6% compared to 1.7±1.0%, Figure 4.5B). Cisplatin at a concentration of 3.16 μg/ml did not induce significant cell death. Based on these results, the maximum concentration in subsequent experiments was set at 3.16 ng/ml for dDAVP and 3.16 μg/ml for cisplatin.

A


*
Control dDAVP
3.16 ng/ml dDAVP
Fold change to control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Apoptosis
Necrosis

B


**
Control
1 ug/ml Cisplatin
3.16 ug/ml Cisplatin
10 ug/ml Cisplatin
Fold change to control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Apoptosis
Necrosis

C

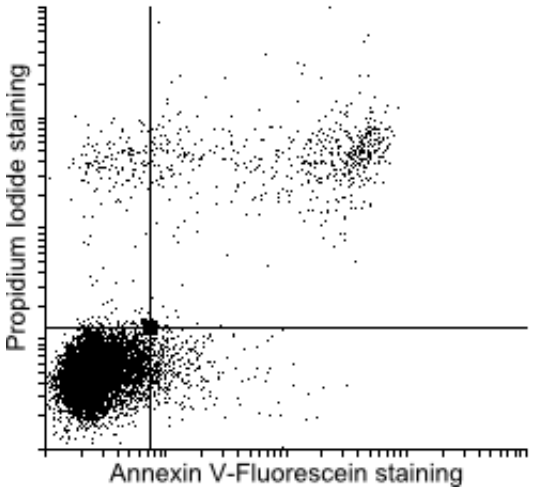


Figure 4.5 Change in the proportion of apoptotic and necrotic cells following stimulation.

Cells were stimulated with A) dDAVP and B) cisplatin. Cells were stained with Annexin V-fluorescein and propidium iodide and indicators of apoptosis and necrosis respectively and then analysed by FACS. Bars are means of 3 experiments. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$. C) A dot plot showing one sample analysed by FACS. Cells in the lower right, upper left and upper right quadrants are considered to be apoptotic, primary necrotic and secondary necrotic respectively.

The mCCD cells were stimulated with dDAVP for 96 hours with the media collected and replaced at 48 hours. Stimulation of the mCCD cells with dDAVP did not alter exosome release as measured by flotillin 1 in the ultracentrifuge pellet after 48 hours or 96 hours. AQP2 was not detected in the exosomes released by the cells after 48 hours. At 96 hours AQP2 was detected in the exosomes from the stimulated cells but not the unstimulated cells (Figure 4.6, 4.7). This appeared to be a dose dependent increase which correlated with the concentration of AQP2 in the cells (Figure 4.8).

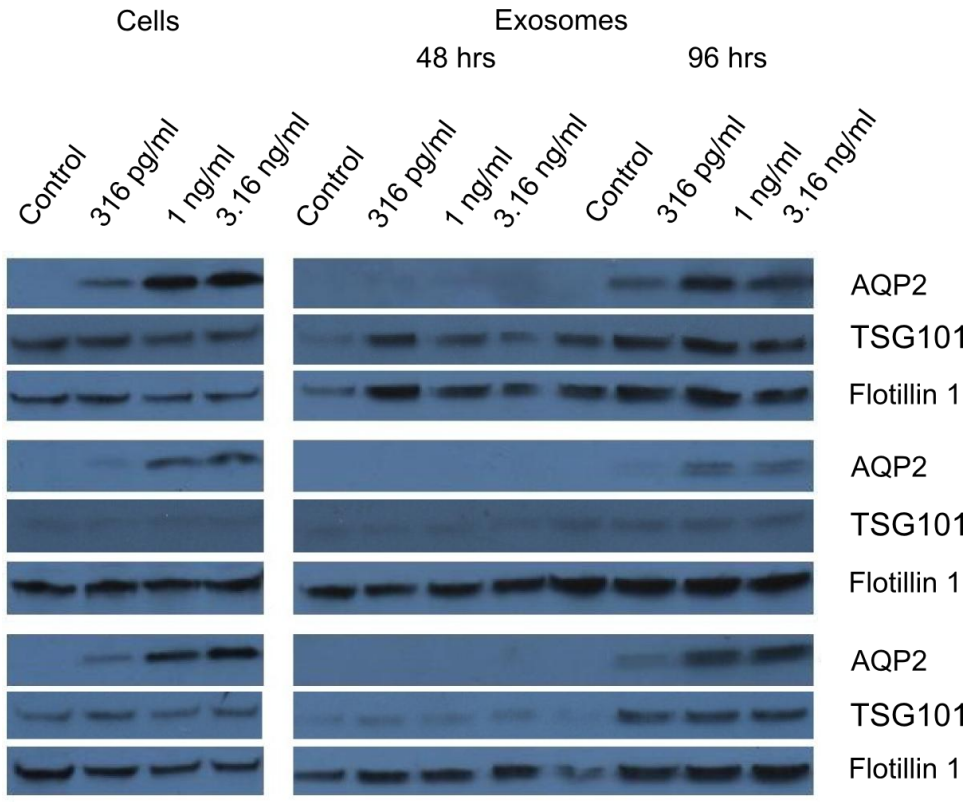
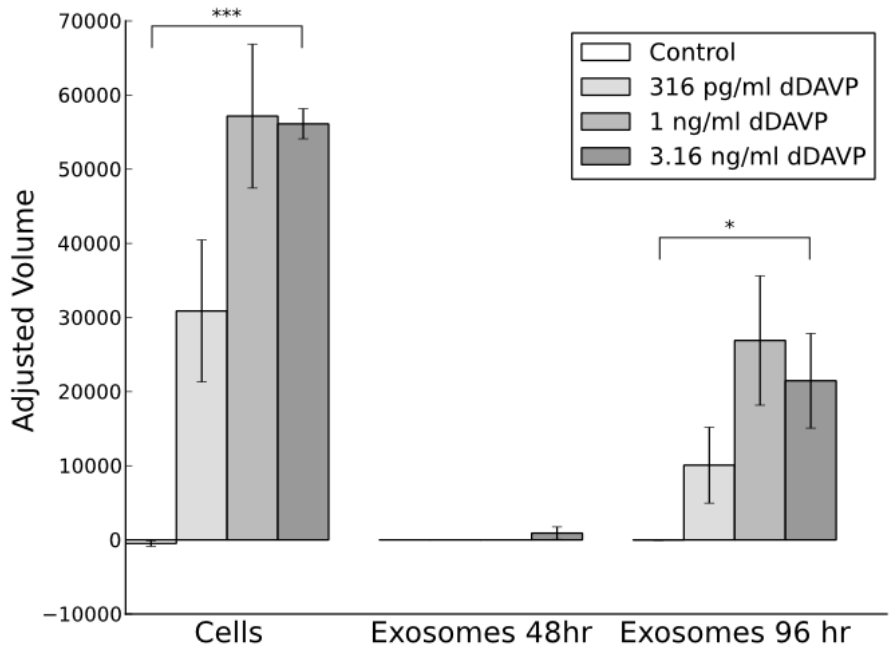


Figure 4.6 Western blot for AQP2, flotillin 1 and TSG101 on exosomes and cell extracts from dDAVP stimulated mCCD cells.
Cell extracts were collected at 96 hours. AQP2 released in exosomes is increased by dDAVP stimulation at 96 hours but not at 48 hours. AQP2 is also upregulated in the cell extracts which were collected at 96 hours. The release of exosomes as measured by flotillin 1 and TSG101 does not change.

A


***
*
Control
316 pg/ml dDAVP
1 ng/ml dDAVP
3.16 ng/ml dDAVP
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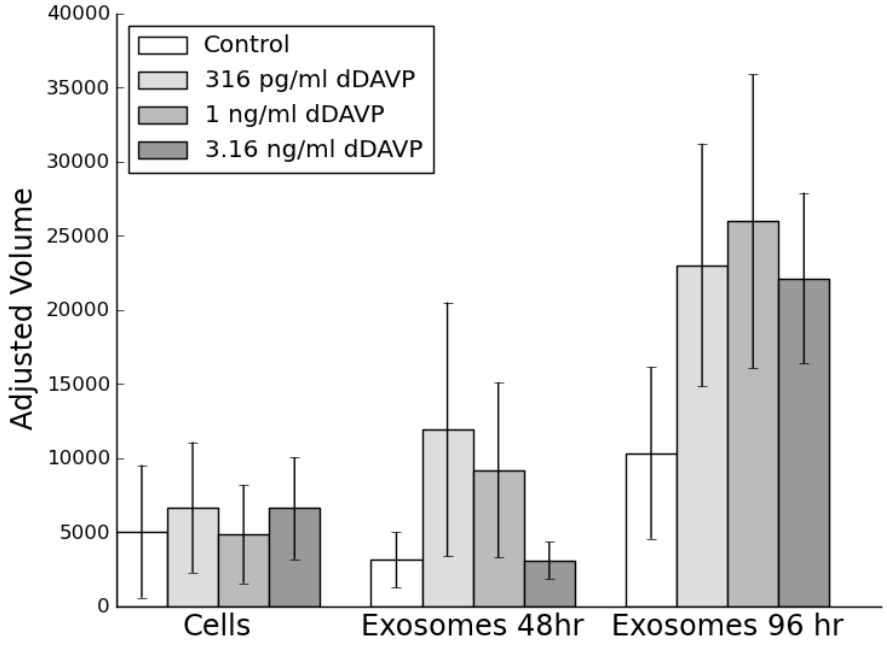
Cells
Exosomes 48hr
Exosomes 96 hr

B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Cells
Exosomes 48hr
Exosomes 96 hr

C

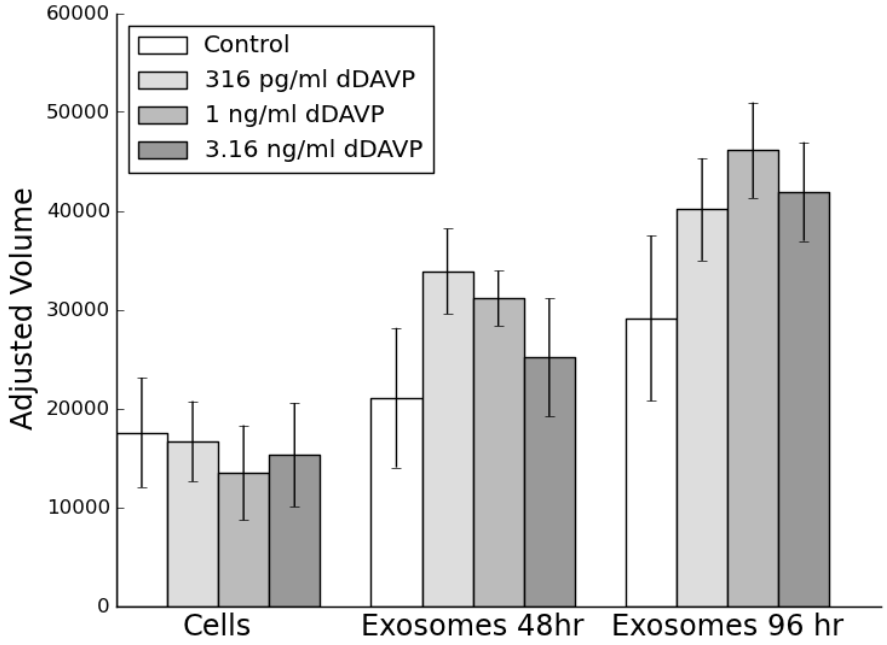


Figure 4.7 Semi-quantitative densitometry on the bands obtained by western blot on exosomes and cell extracts from dDAVP stimulated cells. Densitometry on bands for A) AQP2, B) TSG101 and C) flotillin 1. Cell extracts were collected at 96 hours. AQP2 released in exosomes is increased by dDAVP stimulation at 96 hours but not at 48 hours. AQP2 is also upregulated in the cell extracts which were collected at 96 hours. The release of exosomes as measured by flotillin 1 and TSG101 does not change. * $p < 0.05$, *** $p < 0.001$, n=3.

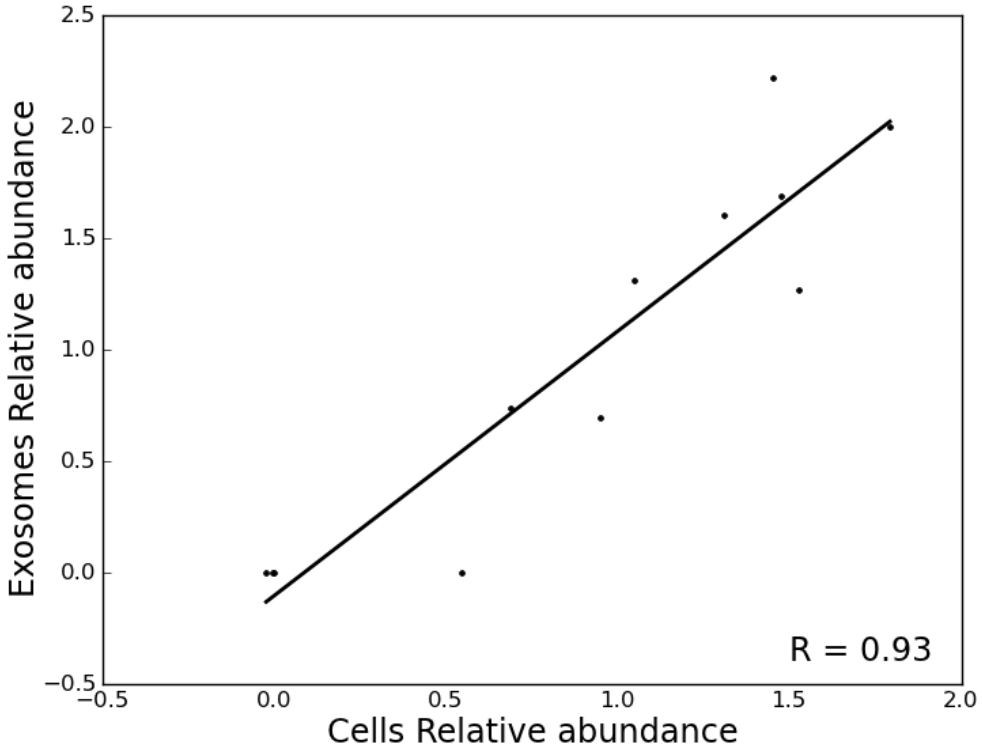


Figure 4.8 Correlation between band intensities for AQP2 in the exosomes and cell extracts of dDAVP stimulated cells.
Band intensities were measured by semi-quantitative densitometry on western blots for AQP2 in the cells and the exosomes following stimulation for 96 hours with dDAVP.

To investigate whether AQP2 was present in the cells at 48 hours a second experiment was conducted with the mCCD cells stimulated with dDAVP and then collected at 48 hours. The exosomal markers TSG101 and flotillin 1 are present in the cells at 48 hours at approximately equivalent levels to 96 hours. AQP2 is only faintly detected even at the highest concentration of dDAVP (Figure 4.9).

To confirm that AQP2 is present in exosomes the constituents of the ultracentrifuge pellet were separated on a density gradient using isopycnic centrifugation. AQP2 co-localised with the exosomal markers TSG101 and flotillin 1 in fractions 3 and 4 (Figure 4.2). This corresponded to a density of 1.10 – 1.15 g.cm-3 which would be expected for exosomes.

Stimulation of the mCCD cells with cisplatin did not alter exosome release as measured by flotillin 1 in the ultracentrifuge pellet. Fetuin A was present in the exosomes released from the unstimulated cells and did not appear to increase with cisplatin treatment (Figures 4.10, 4.11).

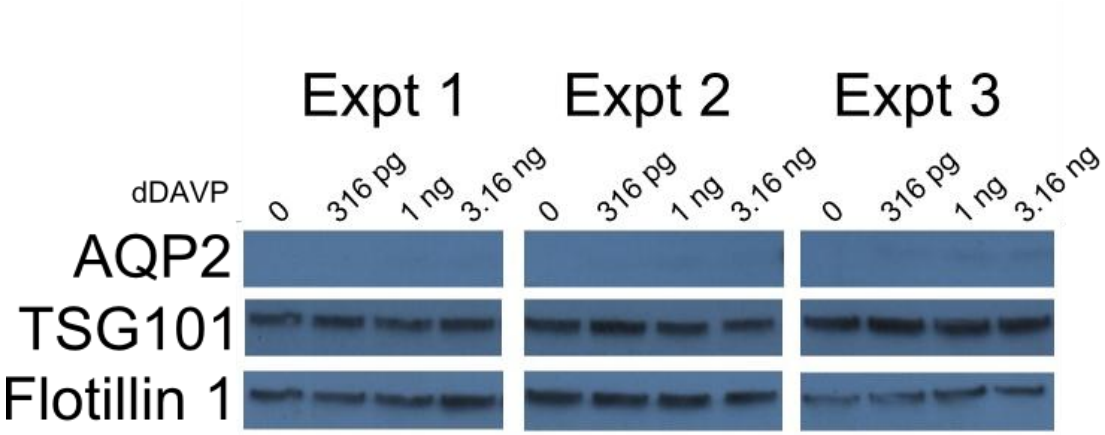


Figure 4.9 Western blot for AQP2, TSG101 and flotillin 1 on cell extracts from mCCD cells stimulated with dDAVP for 48 hours.
AQP2 is only faintly detected in experiment 3 at the highest dose of dDAVP. The exosomal markers TSG101 and flotillin 1 do not change.

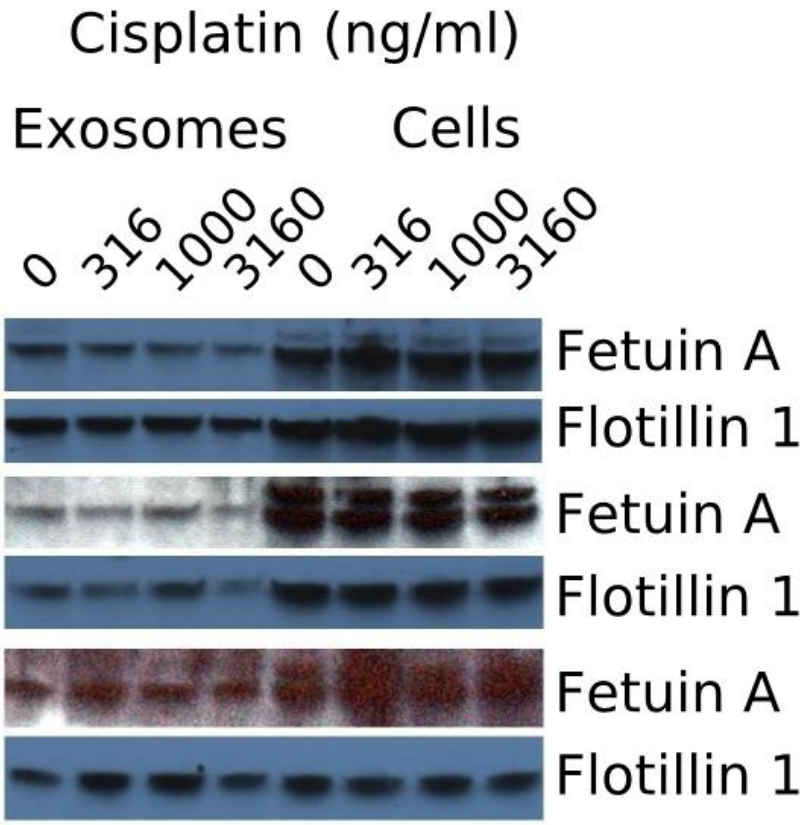
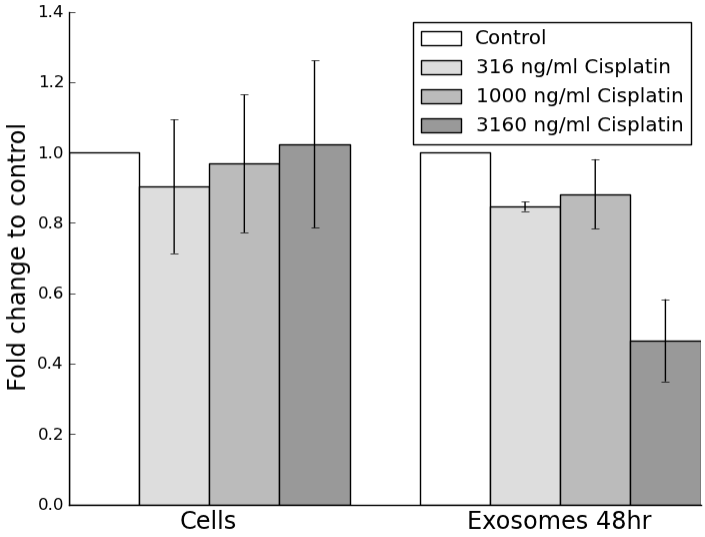


Figure 4.10 Western blot for fetuin A and flotillin 1 on exosomes from cisplatin stimulated mCCD cells.
Fetuin A is present at all doses of cisplatin and does not increase with dose. The release of exosomes as measured by flotillin 1 also does not change. Presented are 3 independent biological replicates.

A



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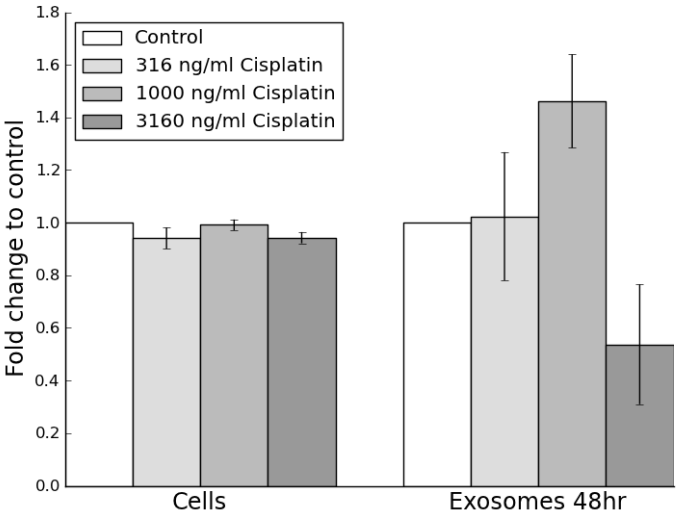


Figure 4.11 Semi-quantitative densitometry on bands obtained by western blot for A) fetuin A and B) flotillin 1 on exosomes from cisplatin stimulated mCCD cells.

Fold changes are on the adjusted volume with respect to the control. Fold changes were used to prevent global changes in band density between repeats obscuring any dose dependent change. Fetuin A is present at all doses of cisplatin and does not increase with dose. The release of exosomes as measured by flotillin 1 also does not change. n=3.

4.3.3. Biological activity of released exosomes

Exosomes can act as intercellular messengers by interacting with the cell surface and/or by being absorbed by cells. To investigate exosome-cell interaction we collected exosomes from cells stimulated with dDAVP for 96 hours and then applied these exosomes back on to mCCD cells. After a 48 hour incubation the presence of AQP2 in the mCCD cells was quantified by western blot and semi-quantitative densitometry (Figure 4.12, 4.13). AQP2 in the cells stimulated with exosomes from dDAVP stimulated cells was increased ($p=0.017$). As controls the increase in AQP2 in cells following dDAVP stimulation was quantified ($p=0.040$) and the presence of AQP2 in the exosomes ($p=0.045$) was confirmed.

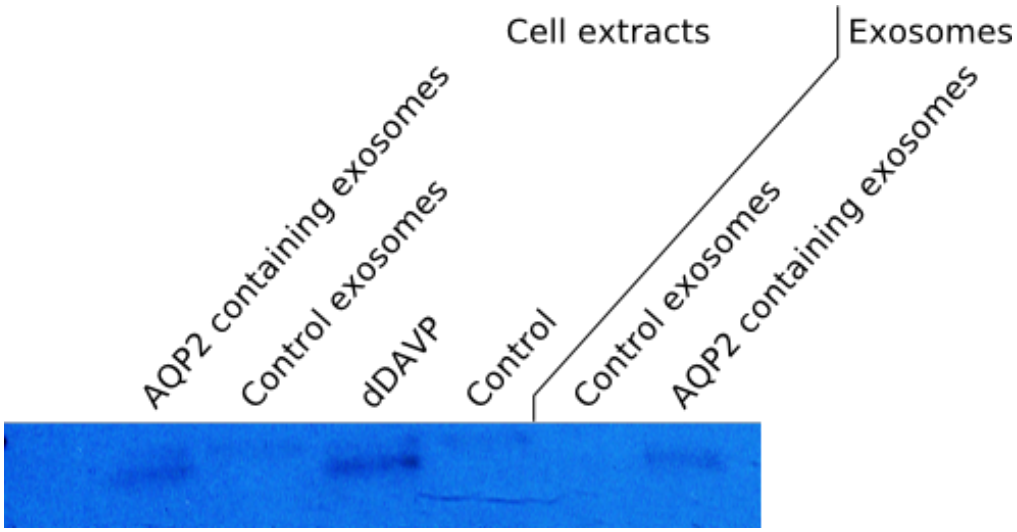


Figure 4.12 Western blot for AQP2 on cell lysates following incubation with AQP2 containing exosomes.
AQP2 is present in the cells extracts following incubation with AQP2 containing exosomes or with dDAVP.

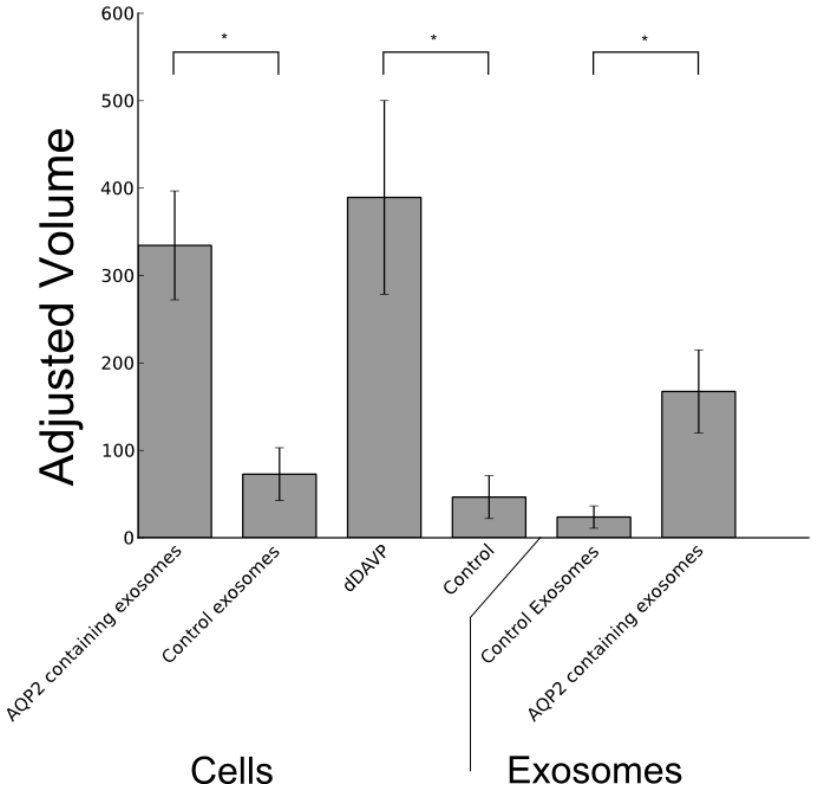


Figure 4.13 Semi-quantitative densitometry on bands obtained by western blot for AQP2 following stimulation of mCCD cells with AQP2 containing exosomes.
* $p<0.05$, n=4.

4.4. Discussion

Exosomes have been demonstrated to be present in human and rodent urine [10, 40, 51, 110, 131, 160]. The complexity of in vivo studies in humans presents significant challenges in understanding the role of urinary exosomes in health and disease. Although problems remain in translating results from in vitro models back to the whole organism they are a useful approach to simplifying an experimental system. This approach has been applied to the study of exosomes with considerable success [18]. Published articles suggest that exosomes are released, to a greater or lesser extent, by all cells. The in vitro model documented here used a cortical collecting duct cell line derived from the kidney of the mouse [118]. Using western blot, isopycnic centrifugation and transmission electron microscopy we have shown that this cell line does release exosomes.

In previous studies ionomycin has been used to induce exosome release [40, 162-164]. Ionomycin is a calcium ionophore which facilitates the influx of calcium into the cell. Ordinarily the concentration of calcium in the cytosol is low. An increase in the cytosolic calcium concentration is a signal for a variety of processes including secretion of the cell contents. In the mCCD cells ionomycin caused a rapid release of exosomes from the cells into the media.

Ionomycin is a generic stimulus of exosome release which will work for many cell types. Focusing more specifically on the mCCD cells two hypotheses surrounding physiological and toxicological stimuli were investigated. Exosomes were first suggested as a possible explanation for the presence of the membrane protein AQP2 in the urine as early as 1995 [165].

The group which established the mCCD cell line also demonstrated that AQP2 was increased in these cells following incubation with dDAVP [161]. The data presented in this chapter demonstrate that AQP2 is increased in the exosomes following stimulation of the cells with dDAVP. Using isopycnic centrifugation and western blot we confirmed that the released AQP2 was present in exosomes.

The presence of AQP2 in the exosomes closely mirrored the presence of AQP2 in the cells. AQP2 was present in neither the exosomes nor the cells prior to stimulation and then present in both following stimulation for 96 hours. The increase in AQP2 concentration in the exosomes faithfully reflected the increase in cellular AQP2.

Exosomal fetuin A has been identified as a potential biomarker of cisplatin-induced acute kidney injury [160]. In our in vitro model stimulation with cisplatin did not alter the presence of fetuin A in the exosomes. The lack in response could be explained by other sections of the nephron being responsible for the increase seen in the rat model. Fetuin A is widely expressed and is present in blood plasma. Therefore, the increase in fetuin A could be due to leakage of fetuin A from the plasma into the urine and subsequent contamination of the exosomal preparation by the soluble proteome of the urine.

The two models investigated here support the hypothesis that changes in the exosomes reflect changes in the cells. Although fetuin A did not change in the exosomes as we had predicted from in vivo findings, fetuin A also failed

to change in the cells. AQP2 in the exosomes increased with stimulation but only after 96 hours which was consistent with the response seen in the cells.

In addition to our interest in exosomes as a potential source of biomarkers exosomes have biological activity in a variety of systems. Exosomes can alter cellular activity by interaction of receptors following contact with the cell surface or uptake of the exosomes and transfer of the exosomal contents to the cell. Incubating AQP2 containing exosomes with cells results in increased AQP2 in the cell lysate. The protocol used would not differentiate between adsorption of exosomes onto the cell surface and uptake of exosomes absorption of exosomes into the cells. These results establish that the exosomes interact with the cells but further work will be needed to characterise the interaction.

The urinary exosomes have been hypothesised to reflect the state of the cells in the kidneys but there has been little robust evidence to support this. Packaging of intracellular proteins into intralumenal vesicles occurs via several pathways, including targeting of monoubiquitinated proteins and association with lipid rafts. It remains unclear what impact these different pathways will have on the hypothesised relationship. Despite this uncertainty we show here that a change in the presence of a protein, AQP2, in the exosomes does appear to reflect a change in the presence of that protein in the mCCD cells. Furthermore, exosomes can transfer AQP2 between cells.

5. Identification of exosomes in human cerebrospinal fluid

5.1. Introduction

Neurological diseases produce a high burden of morbidity and mortality [105, 166]. This burden will increase substantially in the decades ahead, particularly for conditions associated with aging [106]. Early and accurate diagnosis is essential to ensure that patients receive optimal care. For Alzheimer's disease, the prevalence of which is increasing rapidly due to its association with aging [106], early and accurate diagnosis has only begun to be possible in the last few years. As recently as 2000, the diagnostic criteria for Alzheimer's disease involved excluding all other recognised types of dementia rather than the utilisation of any test specific for Alzheimer's disease [167]. The diagnosis now involves structural and molecular neuroimaging and biomarker detection in the cerebrospinal fluid. Despite the progress that has been made there are still aspects of the disease which remain poorly understood and monitoring disease progression is difficult [107].

The material with the greatest potential for biomarker discovery in the context of neurological disorders is brain tissue. Although tissue may be available post mortem the disease in such samples will likely be highly progressed and may not reflect the early stages of the disease. Obtaining a tissue sample from the brain will involve significant safety risks and is therefore often impractical. The cerebrospinal fluid is in constant contact with the brain tissue and as such is likely to be the next best option. Although preferable to the blood the CSF is not without its problems; the volume obtainable is less than with urine and the protein content is lower than blood, giving less potential material to study. The protein that is present is largely the same high abundant proteins present in the circulation

[168, 169]. As with other biofluids, exosomes offer one option to reduce the concentration of the high abundance proteins and focus on the lower abundance, and assumed more biologically interesting, proteins. As yet exosomes have not been identified in human CSF. There is evidence to suggest that human CSF will contain exosomes; a variety of cell types present in the central nervous system have been shown to release exosomes in vitro [34, 35, 164, 170] and exosomes have been identified in the CSF of sheep [36]. Rat cortical primary cultures have been demonstrated to release exosomes [170] and the prion proteins, PrPC (cellular) and PrPSc (scrapie), when expressed in a glial cell line [34] or a mouse neuroblastoma cell line [35], are released in association with exosomes. Furthermore, exosomes may have an important pathophysiological role in Alzheimer's disease. Cleavage of the amyloid precursor protein by the enzyme β-secretase occurs in a subset of endosomes and a fraction of the Aβ protein is released into the extracellular milieu in association with exosomes [32, 171]. Importantly, exosomal markers are enriched in amyloid plaques from the brains of mice [172] and the post-mortem brains of patients with Alzheimer's disease [32].

Although there is circumstantial evidence to suggest that human CSF will contain exosomes, to date there is no published report confirming that they are present. Using the exosome purification protocol established in the urine (chapter 3) the presence of exosomes in human CSF has been investigated.

5.2. Methods

CSF was collected from five patients undergoing thoraco-abdominal aortic aneurysm repair with full ethical approval (REC reference number 08/S1102/59). These patients routinely have a CSF drain inserted as a standard aspect of their clinical management [114]. The CSF was processed using the protocol developed for the urine (Chapter 2.1.3. and 2.2.). Briefly, the CSF was centrifuged at 15,000 x g to pellet cell debris and any large membrane fragments and then the supernatant centrifuged at 200,000 x g to pellet the exosomes. Protease inhibitors and preservative was added to the CSF prior to centrifugation. The protein content of the exosomal preparation was measured using the BCA assay following the manufacturer's instructions. The protocol used to determine whether exosomes were present followed the approach used in the urine and cell culture media. SDS-PAGE and western blot were used to detect exosomal markers in the ultracentrifuge pellet (Chapter 2.3.1 and 4.). The membrane was also stained with Ponceau S solution to visualise the soluble and exosomal proteomes (Chapter 2.4.2.). SDS-PAGE was performed using the Thermo gel system as described in Chapter 2. Isopycnic centrifugation (Chapter 2.5.) and western blot were used to determine the density of the particles containing the exosomal markers. Transmission electron microscopy (TEM) was used to directly visualise the particles present in the ultracentrifuge pellet (Chapter 2.6.1.) and immuno-TEM used to identify flotillin 1 containing particles (Chapter 2.6.2.).

5.3. Results

Ultracentrifugation of human CSF formed a pellet suggesting that there was a low density membrane fraction present in the CSF. Although there is variation between samples all five contained protein (Figure 5.1). Across the five samples the average was 3.45±0.59 μg protein for each millilitre of CSF processed. This was not significantly different compared to 5.17±1.20 μg protein per millilitre in the urine ($p=0.28$, $n=5$ for the CSF and $n=8$ for the urine). SDS-PAGE was performed on a sample of the ultracentrifuge pellet and the whole CSF. The protein was transferred to a PVDF membrane and then stained with Ponceau S stain (Figure 5.2). There is some overlap in the principal bands but there were bands unique to the ultracentrifuge pellet sample suggesting that this is a unique proteome distinct from the soluble proteome. To investigate whether this proteome was exosomal in origin the presence of known exosomal markers was investigated.

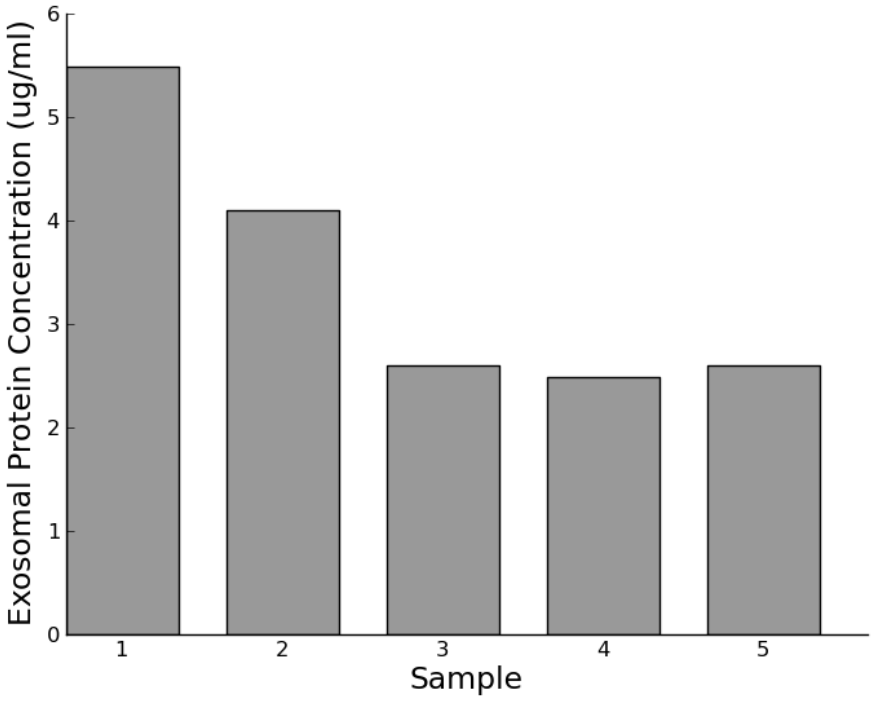


Figure 5.1 Exosomal protein per millilitre of CSF.
The protein in the ultracentrifugation pellet is expressed per ml of CSF. Samples 1 – 5 represent different individuals.

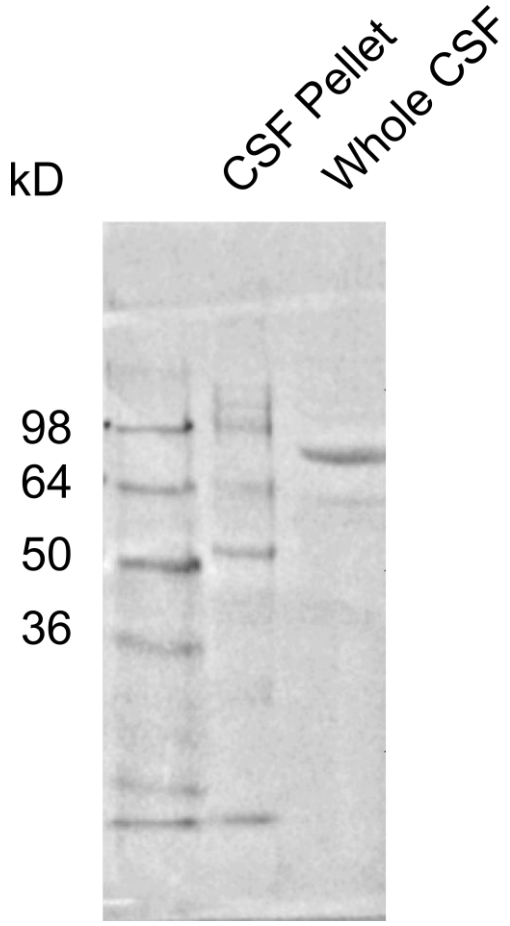


Figure 5.2 SDS-PAGE of the whole human CSF and the ultracentrifuge pellet.
The molecular weight of the main bands in the pellet and whole CSF are different suggesting the proteome of the pellet is different to the soluble protein in the CSF. Presented is data from one representative subject.

Flotillin 1 is associated with lipid rafts which frequently act as the location for exosomal formation and as such flotillin 1 is widely reported to be present in exosomes [1]. TSG101 is a component of the ESCRT-I complex and is involved in the formation of exosomes. TSG101 is also widely reported to be an exosomal marker [173]. Western blot for flotillin 1 and TSG101 on the ultracentrifuge pellet and supernatant showed enrichment of both proteins in the pellet (Figure 5.3).

The density of the flotillin 1 and TSG101 positive particles was determined using isopycnic centrifugation on a sucrose step gradient. Six fractions were collected from the gradient and a western blot for flotillin 1 was performed to determine its location (Figure 5.4). Flotillin 1 localised to fractions 3 and 4 which corresponds to a density of 1.10-1.14 g.cm-3.

Using transmission electron microscopy (TEM) the particles in the ultracentrifuge pellet were directly visualised. In previous studies exosomes have been shown to vary in size from 20 to 100 nm and have a cup-shaped appearance. TEM revealed a number of structures of the correct size and appearance including those highlighted in the electrograph (Figure 5.5). In contrast to other biofluids many of the structures with the characteristic shape for exosomes were at the top of the size range, in the region of 100 nm. As a final step in establishing the presence of exosomes, the TEM grid was incubated with an anti-flotillin 1 antibody followed by a secondary antibody tagged with a 10 nm gold nanoparticle which shows up as a dark dot when viewed under the transmission electron microscope. This demonstrated cup-

shaped structures of the characteristic size for exosomes showing bound gold nanoparticles (Figure 5.6). When the flotillin 1 antibody was replaced with an isotype control antibody although structures of the characteristic size and appearance could be identified none showed bound gold nanoparticles (data not shown).

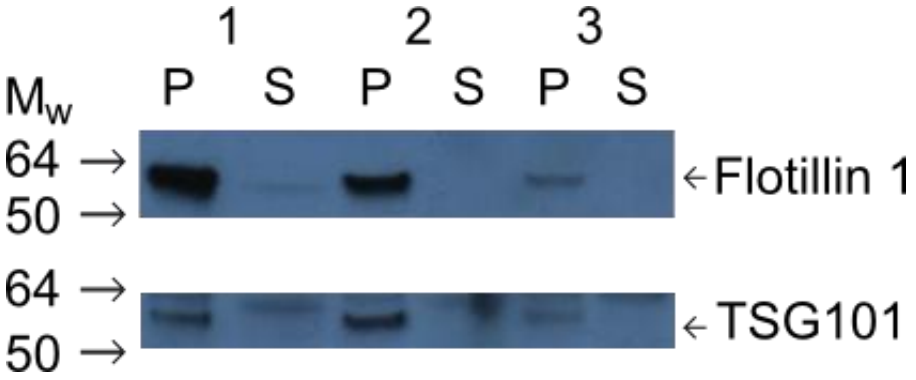


Figure 5.3 Western blot for flotillin 1 and TSG101 on the ultracentrifugation pellet (P) and supernatant (S) for three samples of CSF. Both exosomal markers are enriched in the pellets. 1-3 indicate samples from 3 subjects.

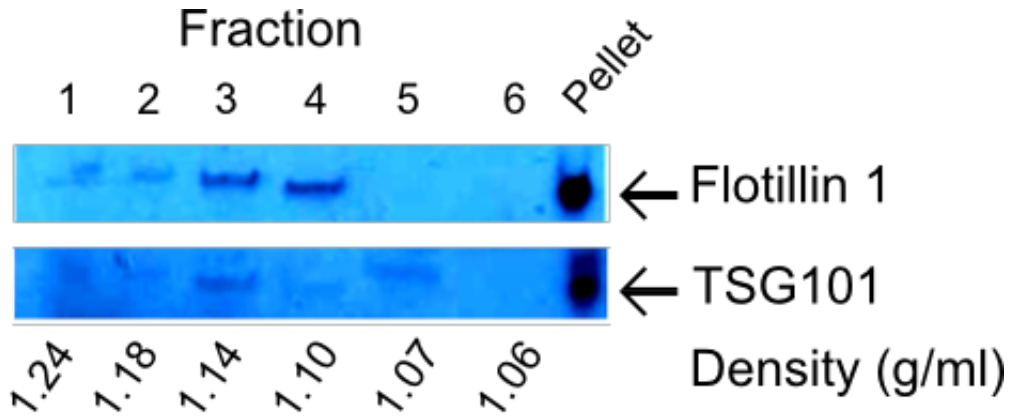


Figure 5.4 Western blot for flotillin 1 and TSG101 on fractions obtained following isopycnic centrifugation of the resuspended ultracentrifuge pellet.

The exosomal markers are present in fractions corresponding to a density of 1.10-1.14 g.cm^{-3} which is consistent with previous reports for exosomes. This distribution has been consistently observed over four replicates. The exosomal markers are present in the range 1.10-1.18 g.cm^{-3} for all repeats.

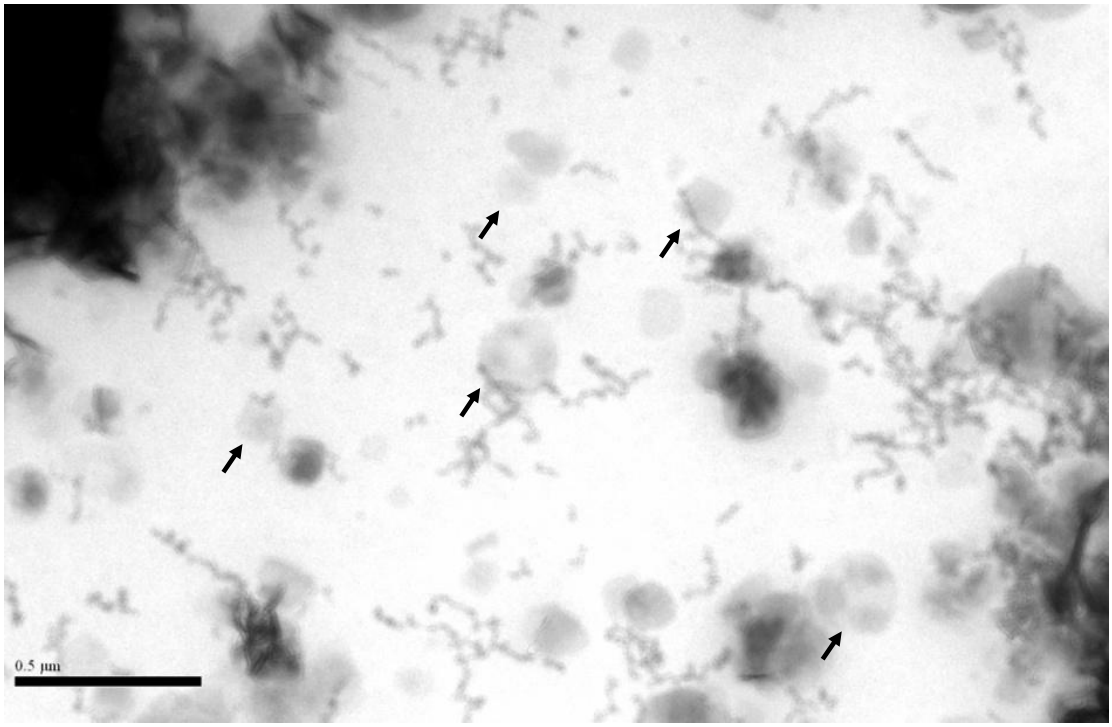


Figure 5.5 Transmission electron micrograph of exosomes derived from human CSF.
Exosomes have previously been described as 20-100 nm in diameter and cup-shaped in appearance. Arrows highlight several structures with size and shape consistent with exosomes.

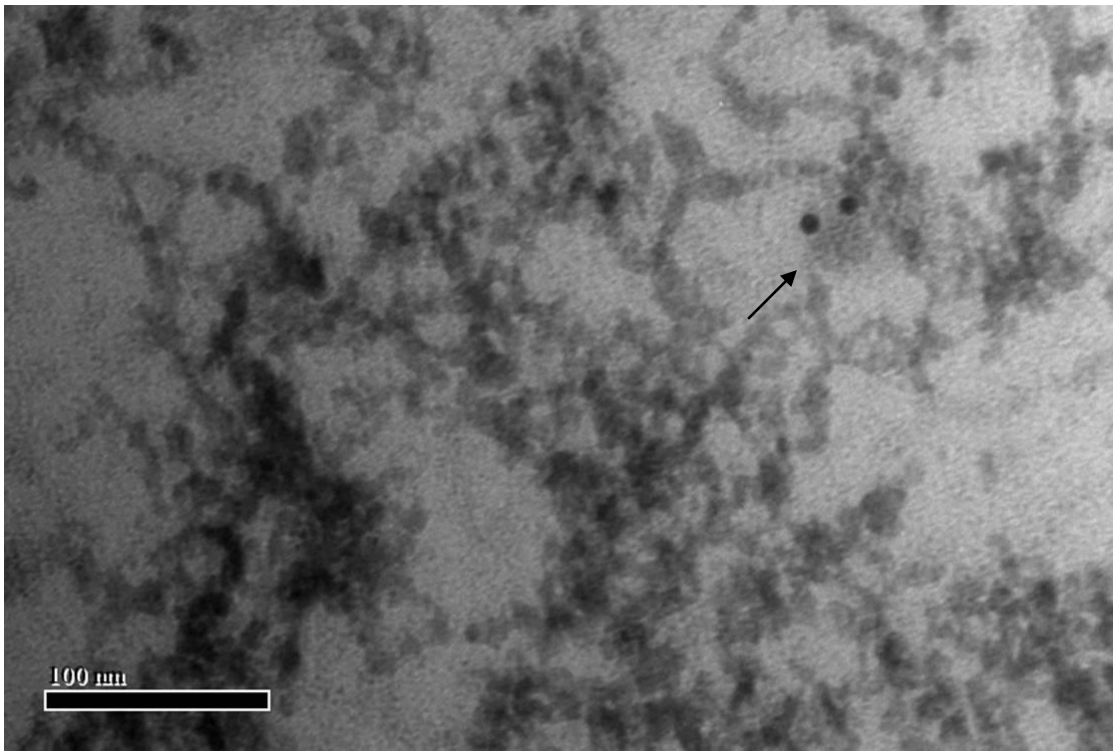


Figure 5.6 Transmission electron micrograph of exosomes immunolabelled for flotillin 1.
Exosomes have previously been described as 20-100 nm in diameter and cup-shaped in appearance. Flotillin 1 is a known exosomal marker. An arrow highlights an exosome displaying two gold nanoparticles consistent with an exosome containing flotillin 1.

5.4. Discussion

Release of exosomes from a variety of different neuronal cell lines has been demonstrated by several research groups. In addition to demonstrating their release [170] these groups have reported that the exosomes possess biological activity, for example in the context of Alzheimer's disease [32, 171], Parkinson's disease [164] and prion diseases [33-35]. In addition to the in vitro studies there has been one study which has found exosomes in the CSF of sheep [36]. Despite the potential importance of exosomes in the context of the central nervous system (CNS) and neurological diseases, no group has reported their presence in human CSF. In the present study we have demonstrated that exosomes are present in, and can be isolated from, the CSF of humans. We have identified exosomes in human CSF using the same approach used for the urine (Chapter 3). The evidence takes three forms: 1) proteins widely documented to be present within exosomes are enriched in the ultracentrifugation pellet in comparison with the supernatant; 2) following separation of the ultracentrifugation pellet based on density, the markers localise to a density range which is consistent with these proteins being contained in exosomes; 3) direct visualisation of the resupended membrane vesicles from the ultracentrifugation pellet revealed the presence of structures matching in size and shape those seen in previous studies of exosomes. In a further experiment these structures were shown to contain the exosomal marker flotillin 1.

Exosomes isolated from the CSF are a potentially valuable source of biomarkers for neurological disease. Based on observations in other, particularly in vitro, systems it is reasonable to hypothesize that the exosomes in the CSF will reflect the state of the cells in the CNS. Although

not as common as taking a blood or urine sample, obtaining a CSF sample is still a relatively routine procedure and a robust panel of biomarkers would be advantageous in the diagnosis and treatment of a variety of neurological diseases [107]. The treatments available for the most significant neurological diseases focus on managing the symptoms and delaying progression of the disease [107, 174]. Early diagnosis will enable the commencement of treatment while the clinical burden of disease is still low. Furthermore, there is some evidence from animal models to suggest that therapies will be more effective when begun early in the progression of the disease [175-177]. Although some treatments are available there is still a significant need for more effective interventions [178, 179]. The effectiveness of potential new therapies will be assessed in clinical trials against a variety of cognitive and functional primary end points [174]. Unfortunately the outcome of such tests is impacted by factors other than disease pathology which may confound the results. A panel of biomarkers might provide a more accurate indication of the effectiveness of an intervention or identify a subpopulation of the trial group with enhanced benefit.

Several groups have presented evidence which suggests that beyond their potential utility as a source of biomarkers exosomes may also have a biological role in a number of neurological diseases. It has previously been shown that alix [32] and flotillin 1 [172], both markers of exosomes, are present in the amyloid plaques which are central to the progression of Alzheimer's disease. This finding is consistent with exosomes, including those present in the CSF, being involved in plaque formation. In vitro cells release prions in association with exosomes and these exosomes are sufficient

to mediate infectivity [33-35]. CSF-derived exosomes from sheep have also been shown to possess prions [36].

Although our findings suggest that CSF-derived exosomes have potential in diagnosing and monitoring neurological disease there are limitations with this study. Firstly, the samples used were obtained from five patients who received a CSF drain as part of standard clinical management for a thoraco-abdominal aortic aneurysm repair operation [114] and to our knowledge none of these patients suffer from any neurological disease. However, exosomes are likely to be present in disease as well as health. Secondly, it remains to be determined whether the exosomes in the CSF represent a mixed population originating from multiple brain regions and cell types, or they are from only a discrete cell type or anatomical site. If the latter is true then it does not necessarily follow that the discrete region or sub-type of cells will overlap with the sub-regions or sub-types involved in a particular neurological disease. Thirdly, because the CSF drain is in place for a prolonged period of time as part of the thoraco-abdominal aortic aneurysm repair surgery a considerable volume of CSF was available for analysis. This was important for the success of this study as the exosomes in the CSF are more dilute than in the urine. For the CSF exosomes to be used as a source of biomarkers it will be first necessary to establish that they can be purified in a useful quantity from a volume more suitable to a diagnostic test.

In summary, this chapter demonstrates that human CSF contains exosomes whose proteome will be investigated in the next chapter.

6. Proteomic analysis of CSF exosomes

6.1. Introduction

There is a high burden of morbidity and mortality associated with neurological diseases [105, 166]. In part this is due to a lack of treatments capable of reversing the underlying disease processes. The currently available treatments focus on alleviating the symptoms and delaying disease progression [167]. Early diagnosis, which will depend on robust biomarkers, is therefore important in reducing the burden of disease. Biomarkers may also be a valuable source of mechanistic information on disease state allowing the subdivision of a patient population and the subsequent targeting of clinical trials and therapies to those patients able to most benefit.

The development of biomarkers is complicated by the difficulty in analysing the central nervous system (CNS). The blood-brain barrier limits passage of proteins from the CNS into the circulation which complicates the study of the plasma as a source of biomarkers. Advanced imaging techniques have transformed the identification and quantification of CNS disease [180], but these approaches are expensive and time consuming. The identification of a protein biomarker in the cerebrospinal fluid (CSF) would potentially offer a more expedient diagnosis at reduced cost and disruption to the patient.

In the previous chapter we demonstrated that exosomes were present in human CSF. We hypothesise that the exosomes within the CSF will provide information on the state of the CNS, as demonstrated with urinary exosomes and kidney disease. There is *in vitro* evidence to support this hypothesis. Studies utilising neuronal and glial cell lines have demonstrated

pathophysiological roles for exosomes in Alzheimer's disease [32, 171], Parkinson's disease [164] and prion diseases [34, 35].

In chapter 3 GeLC-MS/MS identified a number of proteins in human urinary exosomes. Separation of a sample in two dimensions, firstly proteins by denaturing gel electrophoresis and then peptides by liquid chromatography, sufficiently reduced the complexity of the peptide mixture entering the mass spectrometer to allow the analysis of a large variety of different peptides. Analysis of the peptides by tandem mass spectrometry then enabled the accurate identification of many of these peptides and their parent proteins.

GeLC-MS/MS is well suited to identifying many proteins in a sample. For quantifying proteins in a sample the technique is limited due to the use of denaturing gel electrophoresis. The gel slices are cut manually which introduces variability between samples. This variability is increased by differences in peptide recovery between samples. The separation of proteins by gel electrophoresis prior to tryptic digestion can be avoided and an in-solution digestion performed, but this increases the complexity of the sample and a smaller number of proteins will be identified. To keep the complexity of the sample as low as possible, quantitative mass spectrometry was performed on whole protein samples rather than tryptic digests. With this approach identifying the ions detected is not directly possible but the quantitative data should be more complete. To ensure that the mass accuracy was as precise as possible Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) was used. In most other mass spectrometers, for example quadropole and time-of-flight mass spectrometers, ion detection and m/z determination are not fundamentally

linked causing inaccuracies. A quadrupole filters ions in an electric field prior to detection and a time-of-flight mass spectrometer detects ions following selection by flight time. In contrast FT-ICR MS detects ions by their resonance frequency which is directly linked to their m/z ratio [181, 182]. This means that sub-parts per million accuracies are possible with FT-ICR MS.

Due to the potential biological importance of exosomes in the CNS our first objective was to expand on our initial characterisation of the CSF exosomes in chapter 5 by exploring their proteome using GeLC-MS/MS. Prior to searching for biomarkers in the CSF exosomes we need to estimate the variability between individuals. Our second objective was to use label-free quantitative mass spectrometry to assess the variability in the CSF exosomal proteome across people.

6.2. Methods

The same set of CSF samples used in chapter five were used in this study. CSF was collected from five patients undergoing thoraco-abdominal aortic aneurysm repair with full ethical approval. These patients routinely have a CSF drain inserted as a standard aspect of their clinical management [114]. Exosomes were isolated from the CSF by ultracentrifugation as described in chapter 2.2.

GeLC-MS/MS investigation was performed on the exosomes as described in chapter 2.9.1. The proteome was fractionated into 10 fractions by gel electrophoresis prior to in-gel tryptic digestion and LC-MS/MS. The list of identified proteins from each slice was then pooled and duplicates removed.

The immunoglobulin depletion using protein G immobilized on agarose beads and subsequent western blot were performed as described in chapter 2.8.1.

Quantitative LC-FTICR MS (Chapter 2.9.3.) on whole proteins and LC-MS/MS (Chapter 2.9.2.) following in-solution tryptic digestion (Chapter 2.7.2.) was performed on the immunoglobulin depleted exosomal samples. The LC-MS/MS data was processed using the DataAnalysis (Bruker Daltonics, Billerica, MA, USA) software package version 4 SP4. The MASCOT server version 2.2 from Matrix Science[128] was used to identify proteins from the MS/MS data. Mascot searches were performed on the Swissprot 2010 database (519348 sequences) restricted to *Homo sapiens* (20287 sequences) with the following parameters: a specified trypsin cleavage with a maximum of 2 possible missed cleavages, precursor and fragment ion

tolerances of ±1.2 Da and ±0.6 Da respectively, fixed modification caused by carbamidomethylation on cysteines and variable modification by oxidation on methionine. Individual ion spectra were accepted at scores equivalent to $p < 0.05$.

Further removal of the high abundance proteins was attempted using the Agilent Hu-PL7 multiple affinity removal system (MARS) column following the manufacturer's instructions (Chapter 2.8.2.). To verify that the MARS column was functioning correctly a plasma sample was obtained from a healthy 26 year old male. The plasma was prepared for the column following the manufacturer's instructions. Preparation of the exosomal samples was also based on the manufacturer's instructions with some modifications as described in chapter 2.8.2. Protein recovery from the column was assessed using SDS-PAGE as described in chapter 2.3.1. using the invitrogen gel system. As a refinement of the in-solution digest method we used an on-filter digestion protocol which was based on previous reports [183] with some minor modifications as described in chapter 2.7.3.

6.3. Results

6.3.1. GeLC-MS/MS

The CSF exosomal proteome was first investigated using GeLC-MS/MS. The list of identified proteins consisted predominantly of immunoglobulins. Only three other proteins were identified; alpha-2-macroglobulin, filamin A and galectin 3. Querying these three proteins in the Online Mendelian Inheritance in Man (OMIM) database returned entries for the first two proteins linking them to neurological disease. Alpha-2-macroglobulin is involved in Alzheimer's disease where it mediates clearance of amyloid beta [184]. Filamin A is involved in cytoskeletal remodelling and is linked to X-linked periventricular heterotopia in which neurons fail to migrate to the cerebral cortex [185]. Although not linked to a neurological disease in OMIM galectin 3 is a macrophage marker known to be expressed on microglial cells following activation, for example by ischemia [186, 187]. Our patient samples were obtained from a CSF drain during thoraco-abdominal aortic aneurysm repair surgery. CSF drainage is one strategy used during this type of surgery to reduce ischemia which can lead to paraplegia [114] and it is possible that the presence of galectin 3 may reflect low-level ischemia.

6.3.2. Immunoglobulin depletion by protein G

High abundance immunoglobulins limited our ability to identify low abundance proteins. Therefore the exosome containing samples were incubated with protein G immobilized on agarose beads to reduce the amount of immunoglobulin. Western blot for human immunoglobulins identified immunoglobulins ranging from below 36 kDa to above 148 kDa. Strong bands were present in the range 60-100 kDa. After incubation with

protein G immobilized on agarose beads immunoglobulin bands were still present in the 60-100 kDa range but were reduced out with this mass range (Figure 6.1). Exosomes, as measured by the presence of flotillin 1, remained after incubation with the protein G immobilized on agarose beads.

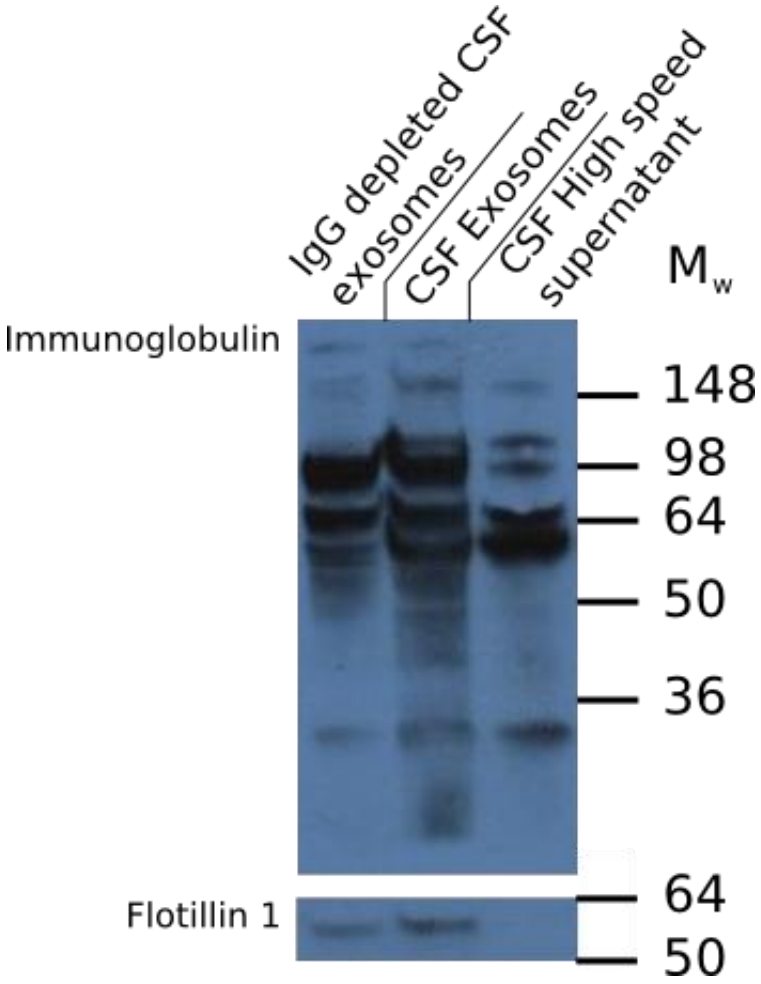


Figure 6.1 Western blot for human immunoglobulin
The supernatant following high speed centrifugation, the purified exosomes and the purified exosomes following depletion of immunoglobulins by incubation with protein G conjugated to agarose beads are shown.

Following immunoglobulin depletion each sample was split into two aliquots. One aliquot was analysed directly by LC-FTICR MS. In-solution digestion was performed on the second aliquot and the resulting peptides were analysed by LC-MS/MS.

6.3.3. LC-MS/MS

In-solution tryptic digestion followed by LC-MS/MS on each sample identified 19 proteins after the removal of duplicates and immunoglobulins (Table 6.1 and Appendix 2). Checking each protein on the exocarta website [122] revealed that ten of the identified proteins had previously been found in exosomes or were closely related to proteins which had previously been found in exosomes. Four proteins were cytosolic, three were nuclear, four were extracellular, four were membrane associated and the remainder were associated with different organelles of the cell. Although not specific several identified proteins are enriched in the CNS. Aquaporin 1 [188], C-reactive protein, lysine-specific demethylase 6A, phosphoglycerate kinase 2 and sodium/iodide cotransporter have all been shown to be elevated in tissues of the CNS during transcriptional studies [189] and serine palmitoyltransferase 3 is involved in the synthesis of sphingolipids which are required for signal transduction[190]

Accession	Name	Mascot score	Sequence coverage (%)	Peptides matched	Protein Mass (kDa)	Previously identified in exosomes	Cellular location
P01023	Alpha-2-macroglobulin	1630	49	66	163	Yes [132, 138]	Cytosol
Q08380	Galectin-3-binding protein	471	33	20	65		Extracellular
P20742	Pregnancy zone protein	202	6	10	164	Yes [34]	Extracellular
P29972	Aquaporin-1	85	7	1	29	Yes [10, 131]	Plasma membrane
P48740	Mannan-binding lectin serine protease 1	71	5	2	79	Yes [138]	Extracellular
P29590	Probable transcription factor PML	51	3	2	97		Cytosol
Q6P995	Protein FAM171B	43	0	3	92		Membrane
Q96RE9	Zinc finger protein 300	43	3	2	69	Related [131, 139]	Nucleus
P00480	Ornithine carbamoyltransferase, mitochondrial	43	2	1	40		Mitochondria
Q86T29	Zinc finger protein 605	43	1	1	74	Related [131, 139]	Nucleus
Q9P2M7	Cingulin	43	0	1	136		Membrane
Q9NUV7	Serine palmitoyltransferase 3	40	5	2	62		Endoplasmic reticulum
Q68D51	DENN domain-containing	40	0	1	106		

	protein 2C						
P02741	C-reactive protein	39	4	1	25		Extracellular
O15550	Lysine-specific demethylase 6A	38	1	2	154		Nucleus
Q8TF72	Shroom 3	38	0	1	217	Related [131]	Cytoskeleton
P36871	Phosphoglucomutase-1	37	2	2	61	Yes [131]	Cytosol
P07205	Phosphoglycerate kinase 2	37	2	1	45	Yes [191]	Cytosol
Q92911	Sodium/iodide cotransporter	37	2	1	69	Yes [134]	Plasma membrane

Table 6.1 Proteins identified in tryptic peptide LC-MS/MS data following depletion of immunoglobulins with protein G conjugated to agarose beads.

6.3.4. LC-FTICR MS

187 distinct proteins were identified in the five samples with 66 present in all five samples (Figure 6.2 and Appendix 3). The coefficient of variation for all proteins present in two or more samples was calculated. The median coefficient of variation was 81% and the 25th percentile was 68% (Figure 6.3). Within the 10 most abundant proteins the maximum coefficient of variation was 14% (Table 6.2).

Variability in retention time complicates the comparison of proteins between samples and is one of the obstacles in label-free quantitative analyses. Manual and, in the latest software packages, automated alignment is necessary for comparison. Particularly amongst the most abundant proteins there was little variability in the retention times which could be adjusted for by the automated alignment (Table 6.2).

The molecular weight of the proteins detected by LC-FT ICR MS are lower than those identified by LC-MS/MS on the tryptic peptides (Figure 6.4). This suggests that each technique provides additional information not available using the other technique.

A B

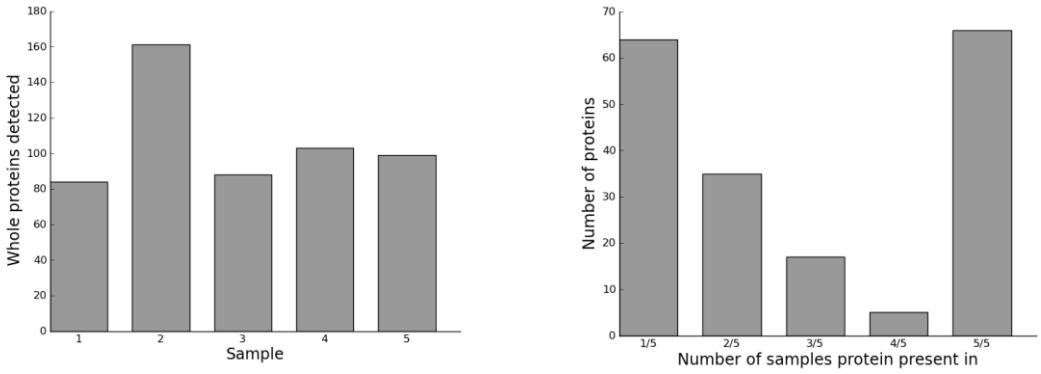


Figure 6.2 Ions detected following LC-FTICR MS on intact proteins from the CSF exosomes after immunoglobulin depletion with protein G conjugated to agarose beads.
A) The number of ions detected in each sample. B) The number of ions detected in different numbers of samples.

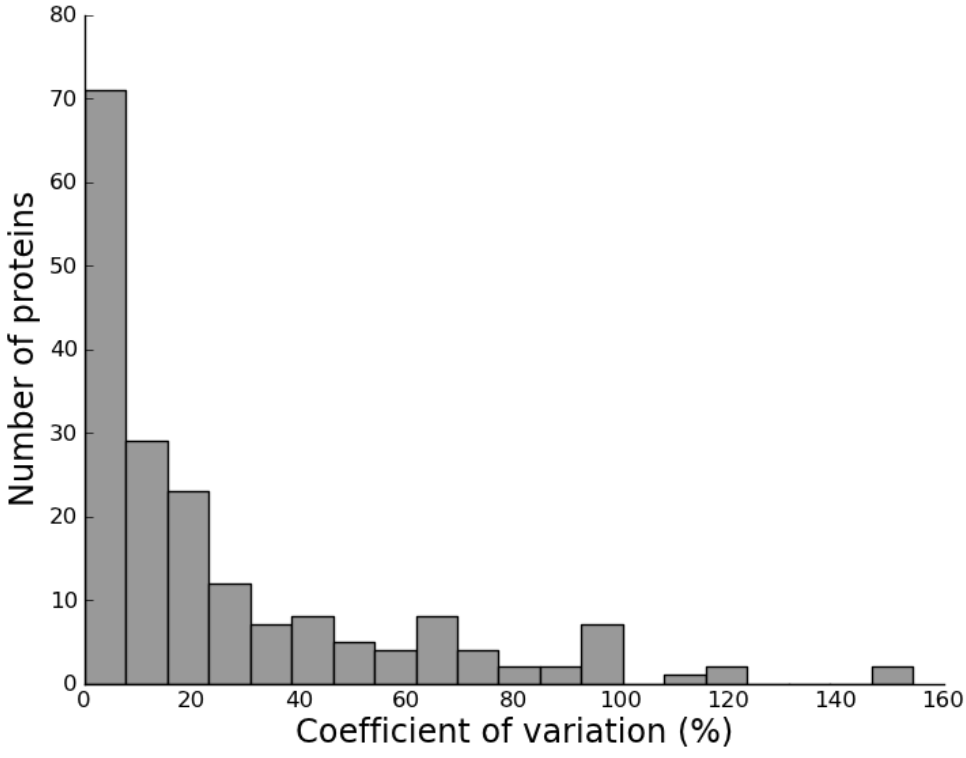


Figure 6.3 Histogram of the coefficient of variation on LC-FT ICR MS protein abundance.

m/z	Charge	Average Abundance	Coefficient of variation	Retention Time (min)				
				Retention 1	Retention 2	Retention 3	Retention 4	Retention 5
659.5042	2	84905.83	9.48	32.35	32.34	32.44	32.41	32.39
637.4901	2	79016.43	8.88	32.35	32.34	32.44	32.41	32.39
681.5181	2	73538.83	10.40	32.35	32.34	32.44	32.41	32.39
615.4762	2	71518.13	7.83	32.28	32.28	32.38	32.35	32.33
703.5339	2	70627.72	10.75	32.42	32.40	32.51	32.47	32.44
593.462	2	64351.47	9.18	32.28	32.28	32.38	32.35	32.33
571.4479	2	51233.77	10.47	32.22	32.21	32.31	32.29	32.28
725.5471	2	45044.52	9.20	32.42	32.40	32.51	32.47	32.44
549.4341	2	41728.51	8.51	32.22	32.21	32.31	32.29	32.28
527.42	2	29260.44	13.92	32.15	32.15	32.24	32.23	32.22

Table 6.2 The ten most abundant proteins detected by LC-FT-ICR MS.

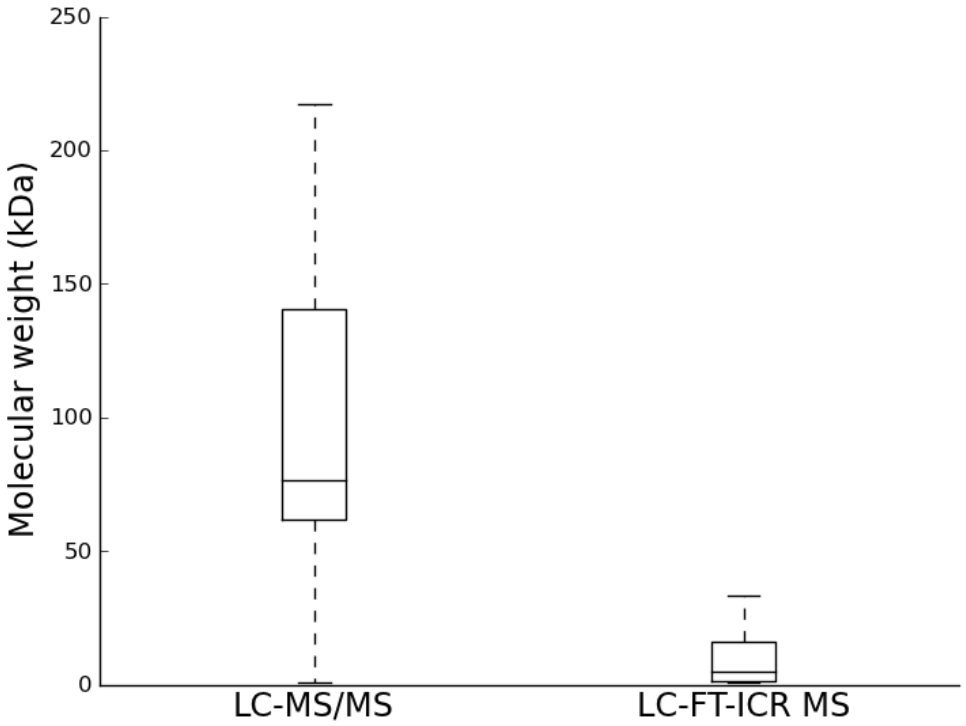


Figure 6.4 Comparison of the molecular weights of the proteins identified by LC-MS/MS and LC-FT ICR MS.

6.3.5. Immunoglobulin depletion by MARS column liquid chromatography

As an alternative to protein G immobilized agarose beads for immunoglobulin removal we used the Agilent HuPL-7 MARS column which is designed to remove the 7 most abundant proteins from human plasma and other biofluids. In early tests we found that our sample was so dilute that we could not adequately recover sufficient protein from the column eluate to analyse on the mass spectrometer (Figure 6.5). To verify that the column was performing correctly we ran the exosomal samples in conjunction with a plasma sample. The UV spectra obtained from running a neat sample of plasma matched the sample spectra given by the manufacturer indicating that the column was functioning correctly and the issue was due to the low concentration (Figure 6.6A). To investigate further the plasma sample was diluted so that the UV peak height matched the peak height for the CSF exosomal sample (Figure 6.6B). The low abundance protein fraction from each sample was then digested using on-filter digestion and the resulting peptides analysed on the LC-MS/MS instrument. In the plasma sample 48 proteins were identified but again no proteins could be identified in the exosomal sample.

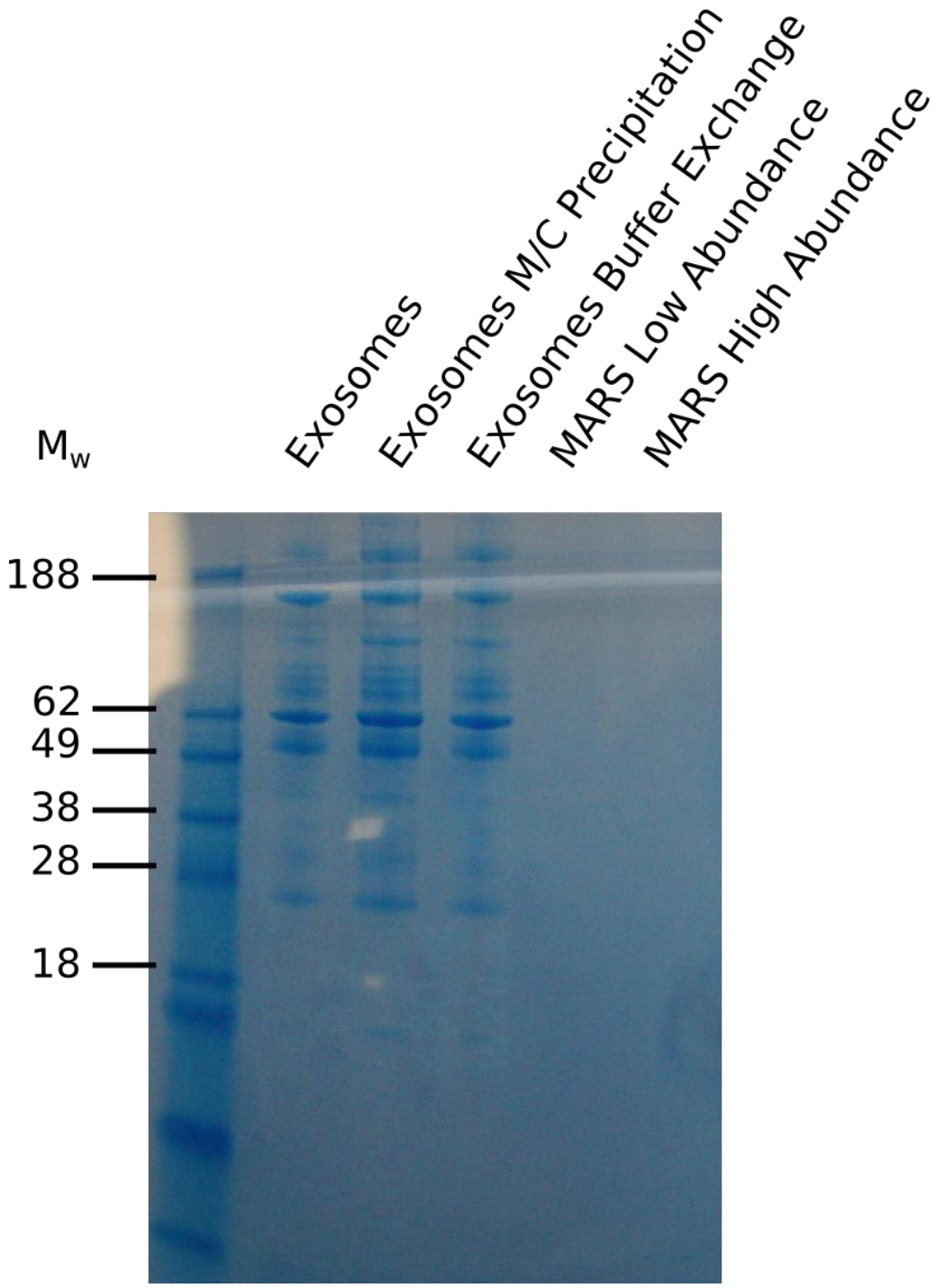


Figure 6.5 SDS-PAGE comparing protein present before and after separation on a MARS column.
Samples are the exosomal preparation, following protein extraction with a methanol/chloroform (M/C) precipitation, following buffer exchange on a molecular weight cut-off filter and then the MARS column eluates for the low and high abundance proteins after concentrating each on a molecular weight cut-off filter.

A B

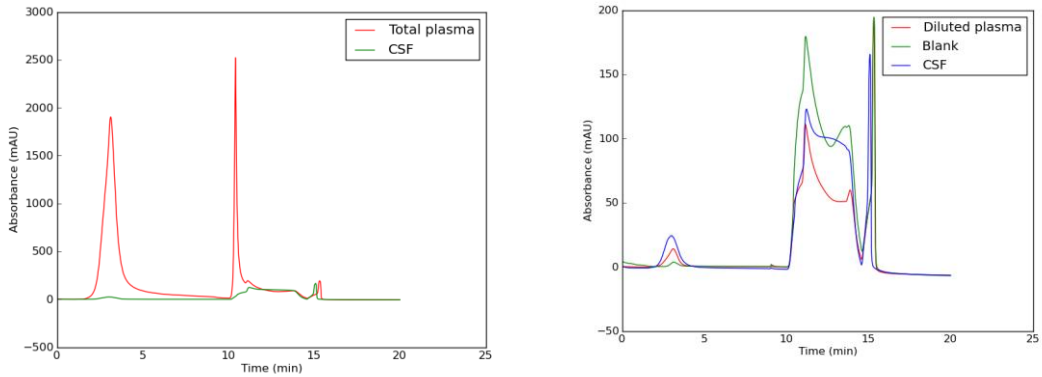


Figure 6.6 UV absorbance on the MARS column eluate.
A) Comparison of a CSF sample and a plasma sample loaded at the concentration recommended by the manufacturer. B) Comparison of a CSF sample, a plasma sample diluted to approximately match the CSF sample and a blank sample.

6.4. Discussion

Using GeLC-MS/MS and LC-MS/MS we have begun cataloguing the proteome of the CSF exosomes identifying 21 distinct proteins in total. We have also used LC-FTICR MS to begin quantifying the components of the CSF exosomal proteome. Comparison of the ions detected and their relative abundance indicates considerable consistency between samples.

GeLC-MS/MS on a sample of CSF exosomes returned information on a smaller number of proteins than had been expected from previous experience with this technique in the urine. The presence of high abundance proteins can mask low abundance proteins and, based on the number of immunoglobulins identified, we concluded this was why so few other proteins were identified. Although the urine also contains a highly abundant protein in uromodulin it is restricted to a narrow mass range. The immunoglobulins in the CSF are present at molecular weights less than 36 kDa to more than 148 kDa as determined by western blot. A polyclonal antibody raised against affinity purified IgG and known to react with IgG, IgA and IgM was used for this western blot. With immunoglobulins present in each slice of the gel they effectively mask the lower abundance proteins.

Our first attempt at resolving this issue was to incubate the exosomes with protein G immobilized on agarose beads. Protein G binds to immunoglobulins in the sample and can be removed from the sample by centrifugation. The exosomes, which have a lower sedimentation coefficient than the beads, remain in the supernatant. This technique did not achieve complete removal of the immunoglobulins but did reduce the mass range

over which immunoglobulins were present. Using this approach quantitative mass spectrometry was performed on whole proteins and on tryptic peptides.

Quantitative LC-FTICR MS indicated substantial similarity between the five samples studied. More than a third of the identified ions were present in all five samples. The ion abundance was also similar between samples with a median coefficient of variation of 81%. This analysis was on five biological replicates without technical replicates. As such, it is not possible to separate out the biological and technical variation from the total variation. The variation observed appears to be approximately comparable to similar previously published studies including one in the whole CSF [108]. Biomarkers are often identified with fold changes sufficiently large to be detected against the background variation in this study. For example Paweletz *et al* identified several ions that changed by more than eight fold with drug treatment [108] and Goonetilleke *et al* identified 34 protein spots altered in pneumococcal meningitis including serotransferrin which was 5.5 fold down regulated in non-survivors [109]. The molecular weights of proteins detected by LC-FT ICR MS and identified by LC-MS/MS on tryptic peptides did not overlap. The proteins detected by LC-FT ICR MS were smaller than those identified by LC-MS/MS. This suggests that these two approaches are complimentary.

Analysis of the tandem mass spectra data on the tryptic peptides identified 18 additional proteins not identified in the GeLC-MS/MS analysis. Although more proteins were identified than in the GeLC-MS/MS experiment the total number was still small in comparison to the urinary proteomics in chapter three and what might be expected from comparison with other exosomal studies. This may in part be due to the use of in-solution digestion rather than in-gel digestion. Following immunoglobulin depletion with the protein

G conjugated agarose beads the immunoglobulins, although still present, were restricted to a narrower range of masses. This suggested that GeLC-MS/MS would limit the immunoglobulins to a small number of the gel slices and the remaining slices would be sufficiently immunoglobulin depleted for the low abundance proteins to be detected. GeLC-MS/MS was not used because it does not work well with quantitative mass spectrometry due to the increased variability introduced due to variable partitioning of proteins into the gel slices, different cut-offs used for the slices, and different recoveries from the slices. In-solution digestion does not have these problems but all the proteins in the sample are mixed together meaning that the immunoglobulins still present overwhelm the low abundance proteins for the entire sample. Alternative separation techniques, such as strong cation exchange and reverse phase liquid chromatography, which are more reproducible than SDS-PAGE may be able to reduce the impact of the immunoglobulins without compromising the quantitative analysis.

With immunoglobulins still detected by LC-MS/MS after incubating exosomes with protein G conjugated agarose beads we used the Agilent MARS Hu-PL7 column as an alternative to remove the immunoglobulins. This column performed well in a recent comparison [192]. It also functioned well in our hands when used on a plasma sample but unfortunately much of the protein in the CSF exosomal samples was lost during processing. It is unclear whether the sample is lost on the MARS column itself or during the concentrating step on the molecular weight cut-off filter after the MARS column. Performing the digestion on the filter did not improve recovery. Due to the insufficient removal of immunoglobulins with the protein G conjugated agarose beads and the poor recovery with the MARS column we are forced to conclude that obtaining a sufficient quantity of exosomal proteins with sufficient purity will require considerably larger volumes of

CSF. Unfortunately the volumes used are already larger than we could reasonably expect to obtain during a biomarker discovery study.

If the CSF exosomes are to be studied as a source of biomarkers then alternative approaches will be needed. It is possible that alternative centrifugation methods such as isopycnic centrifugation or centrifugation on a sucrose cushion [116] may isolate a sufficiently pure sample with small enough losses to render the affinity purification techniques unnecessary. Alternatively the type of study could be changed from discovery proteomics to hypothesis driven proteomics for which a variety of more sensitive mass spectrometry techniques such as multiple reaction monitoring [193, 194] and antibody based techniques such as western blot and ELISA are available. There has also been growing interest in the presence of RNA in exosomes [195, 196]. The proteins present will not interfere with the detection of RNA and the use of RNA biomarkers is therefore another potential alternative.

In summary, we have begun to catalogue the CSF exosomal proteome and quantify the variation between individuals. We have used LC-MS/MS and LC-FT ICR MS which are complimentary approaches in this setting. Improvements in instrument sensitivity and/or sample preparation will be needed before expansion of this study.

7. General Discussion

Robust biomarkers are powerful tools in the detection, diagnosis, monitoring and treatment of disease [197, 198]. We have focused on disease of the kidneys and central nervous system (CNS) as two areas where the need for biomarker development is particularly great. Proteomic analysis of the urine and cerebrospinal fluid (CSF) should provide valuable insights into the state of the kidneys and CNS, respectively. Proteomic analysis of any biofluid is complicated by the high complexity and dynamic range of the constituent proteins [100]. A variety of separation techniques have been developed to facilitate the proteomic analysis of biological samples. Classical techniques separate proteins or peptides along a continuous gradient based on a physical property, for example hydrophobicity in reverse phase liquid chromatography, with the eluate being fed directly to the mass spectrometer or pooled into buckets for further processing. For example two dimensional, and higher, liquid chromatography is gaining increasing use [199-202]. An alternative is to separate a discrete subproteome, for example the phosphoproteome [203, 204] or the glycoproteome [205] which is being studied in cancer proteomics particularly [206]. We have studied exosomes as an alternative subproteome.

The first objective of this thesis was to establish an approach for the purification of exosomes from human urine. Human samples are essential for biomarker validation but for understanding the role of exosomes the complex background inherent in human samples presents a significant challenge. As an alternative, the second objective was to establish an *in vitro* model of exosome release and function in the kidneys.

Exosomes have previously been identified in the urine but so far no study has identified exosomes in the CSF. Our third objective was to investigate the presence of exosomes in the CSF. Our final objective, should exosomes be present in the CSF, was to investigate their proteome.

7.1. Purification strategy

There is considerable variability in the purification strategies used in different studies. Ultracentrifuges have different characteristics. Speed and centrifugation duration can be adjusted to maintain conditions between instruments but this does not seem to be done in many published articles. Exosome purification protocols have been published [46, 116] but they focus on listing working combinations rather than documenting an optimal protocol with instructions on how to adapt the protocol to different ultracentrifuges. The purification protocol and the ultracentrifugation conditions we employed were based on a conservative comparison with previously published protocols but validation of the protocol was still needed. Using exosomal protein markers, density measurement and direct visualisation we have verified that our protocol does purify exosomes from human urine.

Comparison of exosome characteristics from different biofluids or cell types is potentially complicated by the variation in purification protocol. For example, the reported size distribution of exosomes varies between reports potentially due to differences in the centrifugation protocol used. Normalising protocols by k-factor and centrifugation time should minimise protocol variability. Ultrafiltration is another potential approach for exosome purification [47, 207, 208]. This approach is a recent development. If exosome preparations obtained by ultrafiltration and ultracentrifugation are comparable [47] standardising on a ultrafiltration protocol may be less problematic as centrifuge type and speed should have less impact on the purified sample. Such standardisation should facilitate protocol migration to the clinic where access to an ultracentrifuge is unlikely.

7.2. In vitro model

The urine contains exosomes released from the entire length of the nephron [207]. Fluorescence activated cell sorting on exosomes is not possible due to their small size [209] and although partial separation of exosomes from a specific section of the nephron may be possible using immuno-precipitation the resulting sample would be impure and unlikely to contain only the exosomes released by the nephron section of interest. An *in vitro* model avoids the need to isolate a sub-population of the exosomes and facilitates pharmacological modulation. Using a murine cortical collecting duct cell line an *in vitro* model for the study of exosomes in the kidney has been established.

Exosome release was rapidly increased following stimulation with ionomycin. Exosome release following ionomycin stimulation is cell type specific. In the haematopoietic cell line K562 ionomycin increases exosome release [210]. In human podocytes, in contrast to the mCCD cells, no change was observed [40]. This suggests that even within the same organ different mechanisms of release are important. No change in exosome release, as quantified by marker protein, was observed with dDAVP or cisplatin treatment. AQP2 did increase in the exosomes released following dDAVP stimulation and this matched an increase in the cells. This process took 96 hours which was consistent with previous observations in this cell line [161].

Applying exosomes from dDAVP treated cells back on to mCCD cells caused an increase in AQP2 in the cell lysates. Previous studies investigating the interactions between cells and exosomes have found exosomes to both adhere to the surface of cells and to enter cells [195, 211]. The protocol used

in this study is not able to differentiate between AQP2 which has entered the cell and AQP2 which is present in exosomes adhering to the cell surface. Although the wash step prior to collecting the cell lysates could potentially be made more stringent removing more of the adherent exosomes there is no way to be sure that all exosomes on the cell surface have been removed. In previous studies direct visualisation using fluorescence [212] and electron [213, 214] microscopy has been used to identify exosomes both on the cell surface and entering the cell. Combining labelling and direct visualisation can also be used to identify the source and destination of proteins in exosomes. Biotinylation of cell surface proteins can be used to track incorporation into exosomes [215] or measure changes at the plasma membrane following incubation with exosomes [212]. Incorporation of proteins into the plasma membrane can also be tracked by labelling the exosomes [213]. Such techniques powerfully demonstrate entry of exosomes into the cells but quantifying this process is challenging. Sub-cellular fractionation of the cells and subsequent western blot should enable quantification of the AQP2 in the different regions of the cell.

The effect of the exogenously applied exosomes on these cells requires further study. Exosomes have previously been described as conveyors of biological activity and information [1, 16, 42, 173, 216-218] and we are beginning to understand the mechanisms of this transfer. Exosomes share the cell surface markers of their cell of origin and can potentially propagate the same signals. Exosome mediated signalling events are well characterised in the immune system. Depending on their cell of origin exosomes can present major histocompatibility complex (MHC) class I [219], class II [12] and TCR/CD3 complexes [162]. Exosomes can also transfer protein [208, 212,

220] and RNA [195, 221] following cell entry. Valadi *et al* applied exosomes from a mouse cell line on to a human cell line to differentiate transferred proteins from host proteins [195]. Using proteomic analysis proteins originating from the mouse were identified in the cells. These proteins were not identified in the exosomes indicating *de novo* synthesis from exosomal RNA. If the exosomes entered the mCCD cells the increase in AQP2 could potentially be AQP2 protein which was present in the exosomes or *de novo* synthesis of AQP2 from mRNA transcripts carried in the exosomes. Identifying whether transfer of RNA or protein is responsible for the presence of AQP2 will require a method of separating the two sources or blocking one of them. Labelling the exosomes prior to applying them to the cells should differentiate protein transferred in the exosomes from newly translated protein. Alternatively blocking translation would allow the protein transferred to be measured. With a 48 hour incubation translation blockade would have side effects on the cells. Several other studies have used shorter incubation times during which blockade of translation should not negatively effect the cells.

7.3. Exosomes in CSF

We hypothesized that exosomes would be present in human CSF and would be a source of biomarkers for the CNS. Adapting the urinary protocol for the CSF exosomes were identified using western blot for exosomal markers, isopycnic centrifugation and transmission electron microscopy. The potential role of exosomes in the CNS has been discussed previously [222, 223] with their role in communication and signalling highlighted. There is insufficient information available to more than theorise on their activity in healthy tissue [191] but there is evidence of a role in a number of diseases [32, 34, 35, 170-172].

Understanding their proteome will be important in identifying their role in both healthy tissue and disease. Using GeLC-MS/MS and LC-MS/MS we identified 21 proteins. This is a relatively small number of proteins which we attributed to the high abundance of immunoglobulins in the exosomal samples. Attempts to remove the high abundance immunoglobulins were only partially successful with all the protein in the exosomal sample eventually lost. With any purification there is a trade off to be made between purity and recovery. Our attempts to achieve sufficient purity resulted in insufficient recovery for analysis by LC-MS/MS. We were fortunate to have access to large volumes of CSF but the volume of CSF routinely obtainable will be limited so increasing the amount of starting material will not be feasible. If biomarker discovery using the CSF exosomes is to be viable, it will be necessary to either improve the recovery rate or increase the sensitivity and/or specificity of the analysis. At the moment there is no obvious approach to increasing recovery while achieving sufficient purity. Ultrafiltration has been suggested as an alternative to ultracentrifugation but

there is currently no evidence indicating recovery or purity will be improved [47]. Western blot, together with other antibody based technologies, is both more sensitive and specific but is limited in the number of proteins it is possible to investigate. More recently several studies have identified RNA as present in exosomes [195, 224]. A potential mechanism for the inclusion of miRNA in exosomes has been suggested [225, 226] with increasing focus on exosomal miRNA as a source of potential biomarkers [196, 221, 227, 228]. The detection method for RNA, usually PCR or microarray, is insensitive to interference from high abundance proteins and so the need for highly purified exosomal samples is avoided. Although the study design will need to be altered to avoid the issues with high abundance immunoglobulins the CSF exosomes are biologically important and warrant continued study.

7.4. Mass spectrometry

Using GeLC-MS/MS we were able to identify 76 proteins in the urinary exosomes. The urinary exosomes have been well studied [10, 131] with 1132 proteins identified in the latest study [131]. The overlap of our dataset for the urinary exosomal proteome with previous reports was higher than for the CSF exosomal proteome. This would be expected given that the CSF exosomes are novel. The differences between our urinary exosomal proteome dataset and previous reports are still substantial; 23 out of the 76 proteins identified had not been seen in previous reports. This is certainly not unprecedented; a comparison of a literature review and three different proteomics experiments looking at the plasma proteome identified 1,175 proteins of which only 195 (16%) were present in more than one result set [229]. The mass spectrometry techniques used are as yet not able to identify all proteins in a sample and on each repetition will randomly sample different ions leading to new proteins being identified and previously identified proteins being missed. Differences in the databases and algorithms used can also lead to differences in the datasets generated [230]. With a large fraction of identified proteins unique to individual datasets it is likely that a significant proportion of proteins are yet to be identified. Although the urinary exosomal proteome would be expected to be smaller that the plasma proteome at the moment it is dwarfed by the dataset on this more extensively studied proteome [231, 232].

Qualitative mass spectrometry data is valuable in understanding the composition of a sample but additional context can be gained by comparison against other samples. A variety of approaches utilising different labels [73, 76-78] have been developed to enable such comparisons but these

approaches have drawbacks [74, 75]. As an alternative we have explored the ability of label-free mass spectrometry [159] to generate quantitative data in our samples. In contrast to label based techniques, label-free mass spectrometry requires no additional sample processing steps compared to a qualitative study. Label-free mass spectrometry is becoming increasingly viable with improvements in inter-analysis variability and data analysis. The tools necessary to perform the data analysis on these studies are improving rapidly [233]. The data analysis software package used in chapter 3 is a general purpose package and although capable of label-free analysis this was largely a manual process requiring the operator to select matching ions between samples and specify the peaks to be quantified. By comparison the progenesis LC-MS software package used in chapter 6 is specifically designed for label-free quantitative mass spectrometry and is able to automate the alignment and quantitation tasks with the operator supervising and correcting the few erroneous matches. Further work is needed to determine the experimental variability but the results seen with both the manual approach in chapter 3 and the automated approach used in chapter 6 suggest that label-free quantitative mass spectrometry should be a valuable tool during biomarker discovery.

7.5 Future Work

This work could be continued in a number of distinct, though mutually supportive, directions. The purification strategy used was based on a conservative interpretation of the existing literature and subsequent validation in the urine. This purification strategy was inadequate in the CSF with immunoglobulin contamination remaining problematic. A comparison of different ultracentrifugation protocols together with alternatives such as ultrafiltration should be conducted looking at recovery of exosomes and the purity of the resulting sample. Such a study may improve proteomic analysis of the CSF exosomes by increasing the signal (exosomes) and reducing the noise (non-exosomal proteins). Future work should also investigate alternative solutes to sucrose for isopycnic centrifugation and subsequent density determination. Sucrose gradients have been widely used but are time consuming to prepare in comparison to alternatives such as Percoll [207]. As particles will migrate to different densities based on the solute used comparison across studies will not be possible until exosomes have been analysed, side by side, on both gradients.

In the present study AQP2 containing exosomes have been applied to the mCCD cells resulting in an increase in AQP2 in the cell lysate. Whether the exosomes enter the cells or remain attached to the surface remains to be investigated. In published studies, labelling the exosomes and fluorescence microscopy on the cells has been used to demonstrate exosome internalisation [211]. A similar approach could be used for the mCCD cells. Increased AQP2 within the cell may increase water flux across the cells. Water flux, for example as measured by gravimetry [234], is a functional assay amenable to future study.

The mCCD cell line is being used as a model of the collecting duct. A proteomic comparison between urinary and mCCD cell derived exosomes would further validate this *in vitro* model. In addition to CCD derived exosomes the cells of the collecting duct will also be exposed to exosomes shed from cells upstream in the nephron and, possibly, exosomes from infiltrating inflammatory cells. Expanding this model to include other cell types may reveal important data regarding cell to cell signalling.

7.6. Concluding Remarks

Exosomes have a variety of biological roles. Our purification of exosomes in the urine and CSF provides a potentially important target for biomarker discovery and investigation of a novel mechanism of intercellular communication in the kidneys and CNS. The development of the mCCD cell model will facilitate the study of the urinary exosomes. Label-free quantitative mass spectrometry is still a novel technique but has promise for discovery stage proteomic studies.

References

1. Thery, C., M. Ostrowski, and E. Segura, *Membrane vesicles as conveyors of immune responses.* Nat Rev Immunol, 2009. **9**(8): p. 581-93.
2. Simpson, R.J., et al., *Exosomes: proteomic insights and diagnostic potential.* Expert Rev Proteomics, 2009. **6**(3): p. 267-83.
3. Johnstone, R.M., et al., *Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes).* Journal of Biological Chemistry, 1987. **262**(19): p. 9412-9420.
4. Hawari, F.I., et al., *Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: A mechanism for generation of soluble cytokine receptors.* Proceedings of the National Academy of Sciences, 2004. **101**(5): p. 1297-1302.
5. Heijnen, H.F., et al., *Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules.* Blood, 1999. **94**(11): p. 3791-9.
6. Gasser, O., et al., *Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils.* Exp Cell Res, 2003. **285**(2): p. 243-57.
7. Marzesco, A.M., et al., *Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells.* J Cell Sci, 2005. **118**(Pt 13): p. 2849-58.
8. Thery, C., et al., *Proteomic Analysis of Dendritic Cell-Derived Exosomes: A Secreted Subcellular Compartment Distinct from Apoptotic Vesicles.* The Journal of Immunology, 2001. **166**(12): p. 7309-7318.
9. Hanson, P.I., S. Shim, and S.A. Merrill, *Cell biology of the ESCRT machinery.* Curr Opin Cell Biol, 2009. **21**(4): p. 568-74.
10. Pisitkun, T., R.-F. Shen, and M.A. Knepper, *Identification and proteomic profiling of exosomes in human urine.* Proceedings of the National Academy of Sciences, 2004. **101**(36): p. 13368-13373.
11. de Gassart, A., et al., *Lipid raft-associated protein sorting in exosomes.* Blood, 2003. **102**(13): p. 4336-4344.
12. Gauvreau, M.E., et al., *Sorting of MHC class II molecules into exosomes through a ubiquitin-independent pathway.* Traffic, 2009. **10**(10): p. 1518-27.
13. Escola, J.-M., et al., *Selective Enrichment of Tetraspan Proteins on the Internal Vesicles of Multivesicular Endosomes and on Exosomes Secreted by*

Human B-lymphocytes. Journal of Biological Chemistry, 1998. **273**(32): p. 20121-20127.
14. Lakkaraju, A. and E. Rodriguez-Boulan, *Itinerant exosomes: emerging roles in cell and tissue polarity.* Trends Cell Biol, 2008. **18**(5): p. 199-209.
15. Simons, M. and G. Raposo, *Exosomes--vesicular carriers for intercellular communication.* Curr Opin Cell Biol, 2009. **21**(4): p. 575-81.
16. Denzer, K., et al., *Exosome: from internal vesicle of the multivesicular body to intercellular signaling device.* Journal of Cell Science, 2000. **113**(19): p. 3365-3374.
17. Raiborg, C., T.E. Rusten, and H. Stenmark, *Protein sorting into multivesicular endosomes.* Current Opinion in Cell Biology, 2003. **15**(4): p. 446-455.
18. Keller, S., et al., *Exosomes: From biogenesis and secretion to biological function.* Immunology Letters, 2006. **107**(2): p. 102-108.
19. Schorey, J.S. and S. Bhatnagar, *Exosome function: from tumor immunology to pathogen biology.* Traffic, 2008. **9**(6): p. 871-81.
20. van Niel, G., et al., *Exosomes: A Common Pathway for a Specialized Function.* Journal of Biochemistry, 2006. **140**(1): p. 13-21.
21. Greco, V., M. Hannus, and S. Eaton, *Argosomes: a potential vehicle for the spread of morphogens through epithelia.* Cell, 2001. **106**(5): p. 633-45.
22. Cadigan, K.M., *Regulating morphogen gradients in the Drosophila wing.* Seminars in Cell and Developmental Biology, 2002. **13**(2): p. 83-90.
23. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* Journal of Experimental Medicine, 1996. **183**(3): p. 1161-72.
24. Zitvogel, L., E. Angevin, and T. Tursz, *Dendritic cell-based immunotherapy of cancer.* Ann Oncol, 2000. **11 Suppl 3**: p. 199-205.
25. Viaud, S., et al., *Dendritic Cell-Derived Exosomes for Cancer Immunotherapy: What's Next?* Cancer Research, 2010. **70**(4): p. 1281-1285.
26. Andreola, G., et al., *Induction of Lymphocyte Apoptosis by Tumor Cell Secretion of FasL-bearing Microvesicles.* The Journal of Experimental Medicine, 2002. **195**(10): p. 1303-1316.
27. Abusamra, A.J., et al., *Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis.* Blood Cells, Molecules, and Diseases, 2005. **35**(2): p. 169-173.
28. Liu, C., et al., *Murine Mammary Carcinoma Exosomes Promote Tumor Growth by Suppression of NK Cell Function.* The Journal of Immunology, 2006. **176**(3): p. 1375-1385.
29. Van Niel, G., et al., *Intestinal Epithelial Cells Secrete Exosome-like Vesicles.* Gastroenterology, 2001. **121**(2): p. 337-349.

30. Taylor, D.D., S. Akyol, and C. Gercel-Taylor, *Pregnancy-Associated Exosomes and Their Modulation of T Cell Signaling.* The Journal of Immunology, 2006. **176**(3): p. 1534-1542.
31. Toth, B., et al., *Microparticles and exosomes: impact on normal and complicated pregnancy.* Am J Reprod Immunol, 2007. **58**(5): p. 389-402.
32. Rajendran, L., et al., *Alzheimer's disease beta-amyloid peptides are released in association with exosomes.* Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11172-7.
33. Porto-Carreiro, I., et al., *Prions and exosomes: From PrPc trafficking to PrPsc propagation.* Blood Cells, Molecules, and Diseases, 2005. **35**(2): p. 143-148.
34. Fevrier, B., et al., *Cells release prions in association with exosomes.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(26): p. 9683-9688.
35. Alais, S., et al., *Mouse neuroblastoma cells release prion infectivity associated with exosomal vesicles.* Biol Cell, 2008. **100**(10): p. 603-15.
36. Vella, L.J., et al., *Enrichment of prion protein in exosomes derived from ovine cerebral spinal fluid.* Vet Immunol Immunopathol, 2008. **124**(3-4): p. 385-93.
37. Thery, C., et al., *Molecular Characterization of Dendritic Cell-derived Exosomes: Selective Accumulation of the Heat Shock Protein hsc73.* The Journal of Cell Biology, 1999. **147**(3): p. 599-610.
38. Dimitris, S., et al., *Immunoregulatory properties of mast cell-derived exosomes.* Molecular Immunology, 2002. **38**(16-18): p. 1359-1362.
39. Caby, M.-P., et al., *Exosomal-like vesicles are present in human blood plasma.* International Immunology, 2005. **17**(7): p. 879-887.
40. Keller, S., et al., *CD24 is a marker of exosomes secreted into urine and amniotic fluid.* Kidney Int, 2007. **72**(9): p. 1095-102.
41. Bachy, I., R. Kozyraki, and M. Wassef, *The particles of the embryonic cerebrospinal fluid: How could they influence brain development?* Brain Research Bulletin, 2008. **75**(2-4): p. 289-294.
42. Thery, C., L. Zitvogel, and S. Amigorena, *Exosomes: Composition, Biogenesis and Function.* Nature Reviews Immunology, 2002. **2**(8): p. 569-579.
43. Andre, F., et al., *Malignant effusions and immunogenic tumour-derived exosomes.* The Lancet, 2002. **360**(9329): p. 295-305.
44. Ogawa, Y., et al., *Exosome-Like Vesicles with Dipeptidyl Peptidase IV in Human Saliva.* Biological & Pharmaceutical Bulletin, 2008. **31**(6): p. 1059-1062.

45. Prado, N., et al., *Exosomes from Bronchoalveolar Fluid of Tolerized Mice Prevent Allergic Reaction.* The Journal of Immunology, 2008. **181**(2): p. 1519-1525.
46. Gonzales, P.A., et al., *Isolation and purification of exosomes in urine.* Methods Mol Biol, 2010. **641**: p. 89-99.
47. Rood, I.M., et al., *Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome.* Kidney Int, 2010. **78**(8): p. 810-6.
48. Pan, B.-T., et al., *Electron Microscopic Evidence for Externalization of the Transferrin Receptor in Vesicular Form in Sheep Reticulocytes.* The Journal of Cell Biology, 1985. **101**(3): p. 942-948.
49. Tyers, M. and M. Mann, *From genomics to proteomics.* Nature, 2003. **422**(6928): p. 193-197.
50. Anderson, L. and J. Seilhamer, *A comparison of selected mRNA and protein abundances in human liver.* Electrophoresis, 1997. **18**(3-4): p. 533-7.
51. Hoorn, E.J., et al., *Prospects for urinary proteomics: Exosomes as a source of urinary biomarkers (Review Article).* Nephrology, 2005. **10**(3): p. 283-290.
52. Gygi, S.P., et al., *Correlation between protein and mRNA abundance in yeast.* Mol Cell Biol, 1999. **19**(3): p. 1720-30.
53. Blackstock, W.P. and M.P. Weir, *Proteomics: quantitative and physical mapping of cellular proteins.* Trends Biotechnol, 1999. **17**(3): p. 121-7.
54. Aebersold, R. and M. Mann, *Mass spectrometry-based proteomics.* Nature, 2003. **422**(6928): p. 198-207.
55. Domon, B. and R. Aebersold, *Mass Spectrometry and Protein Analysis.* Science, 2006. **312**(5771): p. 212-217.
56. Lee, H.J., A.W. Wark, and R.M. Corn, *Microarray methods for protein biomarker detection.* Analyst, 2008. **133**(8): p. 975-83.
57. Eng, J.K., A.L. McCormack, and J.R. Yates, *An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database.* Journal of the American Society for Mass Spectrometry, 1994. **5**(11): p. 976-989.
58. O'Farrell, P.H., *High resolution two-dimensional electrophoresis of proteins.* Journal of Biological Chemistry, 1975. **250**(10): p. 4007-4021.
59. Timms, J.F. and R. Cramer, *Difference gel electrophoresis.* Proteomics, 2008. **8**(23-24): p. 4886-97.
60. Fenn, J.B., *Electrospray wings for molecular elephants (Nobel lecture).* Angew Chem Int Ed Engl, 2003. **42**(33): p. 3871-94.
61. Cook, K.D., *ASMS members John Fenn and Koichi Tanaka share Nobel: the world learns our "secret".* Journal of the American Society for Mass Spectrometry, 2002. **13**(12): p. 1359-1359.

62. Fenn, J.B., et al., *Electrospray Ionization for Mass Spectrometry of Large Biomolecules.* Science, 1989. **246**(4926): p. 64-71.
63. Whitehouse, C.M., et al., *Electrospray interface for liquid chromatographs and mass spectrometers.* Analytical Chemistry, 1985. **57**(3): p. 675-679.
64. Craig, R., J.P. Cortens, and R.C. Beavis, *The use of proteotypic peptide libraries for protein identification.* Rapid Commun Mass Spectrom, 2005. **19**(13): p. 1844-50.
65. Ong, S.-E. and M. Mann, *Mass spectrometry-based proteomics turns quantitative.* Nat Chem Biol, 2005. **1**(5): p. 252-262.
66. Mallick, P., et al., *Computational prediction of proteotypic peptides for quantitative proteomics.* Nat Biotech, 2007. **25**(1): p. 125-131.
67. Tang, H., et al., *A computational approach toward label-free protein quantification using predicted peptide detectability.* Bioinformatics, 2006. **22**(14): p. e481-488.
68. Lu, P., et al., *Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation.* Nat Biotech, 2007. **25**(1): p. 117-124.
69. Wu, W.W., et al., *Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF.* J Proteome Res, 2006. **5**(3): p. 651-8.
70. Collier, T.S., et al., *Direct comparison of stable isotope labeling by amino acids in cell culture and spectral counting for quantitative proteomics.* Anal Chem, 2010. **82**(20): p. 8696-702.
71. Ryu, S., et al., *Comparison of a label-free quantitative proteomic method based on Peptide ion current area to the isotope coded affinity tag method.* Cancer Inform, 2008. **6**: p. 243-55.
72. Geiger, T., et al., *Super-SILAC mix for quantitative proteomics of human tumor tissue.* Nat Methods, 2010. **7**(5): p. 383-5.
73. Ong, S.-E., et al., *Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics.* Molecular Cellular Proteomics, 2002. **1**(5): p. 376-386.
74. Fenselau, C., *A review of quantitative methods for proteomic studies.* Journal of Chromatography B, 2007. **855**(1): p. 14-20.
75. Bantscheff, M., et al., *Quantitative mass spectrometry in proteomics: a critical review.* Anal Bioanal Chem, 2007. **389**(4): p. 1017-31.
76. Gygi, S.P., et al., *Quantitative analysis of complex protein mixtures using isotope-coded affinity tags.* Nat Biotech, 1999. **17**(10): p. 994-999.
77. Choe, L., et al., *8-plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease.* Proteomics, 2007. **7**(20): p. 3651-60.

78. Pierce, A., et al., *Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases.* Mol Cell Proteomics, 2008. **7**(5): p. 853-63.
79. Ross, P.L., et al., *Multiplexed Protein Quantitation in Saccharomyces cerevisiae Using Amine-reactive Isobaric Tagging Reagents.* Molecular Cellular Proteomics, 2004. **3**(12): p. 1154-1169.
80. Jaitly, N., et al., *Robust algorithm for alignment of liquid chromatography-mass spectrometry analyses in an accurate mass and time tag data analysis pipeline.* Anal Chem, 2006. **78**(21): p. 7397-409.
81. Atkinson, A.J., et al., *Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework*.* Clin Pharmacol Ther, 2001. **69**(3): p. 89-95.
82. Stiles, K.P., et al., *Renal Biopsy in High-Risk Patients With Medical Diseases of the Kidney.* American Journal of Kidney Diseases, 2000. **36**(2): p. 419-433.
83. Fine, D.M., et al., *Diagnostic utility and safety of transjugular kidney biopsy in the obese patient.* Nephrology Dialysis Transplantation, 2004. **19**(7): p. 1798-1802.
84. Star, R.A., *Treatment of acute renal failure.* Kidney Int, 1998. **54**(6): p. 1817-1831.
85. Schrier, R.W. and W. Wang, *Acute Renal Failure and Sepsis.* The New England Journal of Medicine, 2004. **351**(2): p. 159-169.
86. Anderson, R.J.M.D., *Kidney in sepsis *. [Editorial].* Critical Care Medicine, 2007. **35**(9): p. 2223-2224.
87. Bellomo, R., et al., *Acute renal failure – definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group.* Critical Care, 2004. **8**(4): p. R204 - R212.
88. Moran, S.M. and B.D. Myers, *Course of acute renal failure studied by a model of creatinine kinetics.* Kidney International, 1985. **27**(6): p. 928-37.
89. Vaidya, V.S., M.A. Ferguson, and J.V. Bonventre, *Biomarkers of acute kidney injury.* Annu Rev Pharmacol Toxicol, 2008. **48**: p. 463-93.
90. Waikar, S.S. and J.V. Bonventre, *Biomarkers for the diagnosis of acute kidney injury.* Curr Opin Nephrol Hypertens, 2007. **16**(6): p. 557-64.
91. Nickolas, T.L., J. Barasch, and P. Devarajan, *Biomarkers in acute and chronic kidney disease.* Curr Opin Nephrol Hypertens, 2008. **17**(2): p. 127-132.
92. Trof, R.J., et al., *Biomarkers of acute renal injury and renal failure.* Shock, 2006. **26**(3): p. 245-53.
93. Mishra, J., et al., *Identification of Neutrophil Gelatinase-Associated Lipocalin as a Novel Early Urinary Biomarker for Ischemic Renal Injury.*

Journal of the American Society of Nephrology, 2003. **14**(10): p. 2534-2543.
94. Westhuyzen, J., et al., *Measurement of tubular enzymuria facilitates early detection of acute renal impairment in the intensive care unit.* Nephrol Dial Transplant, 2003. **18**(3): p. 543-51.
95. Herget-Rosenthal, S., et al., *Early detection of acute renal failure by serum cystatin C.* Kidney Int, 2004. **66**(3): p. 1115-22.
96. Ichimura, T., et al., *Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury.* J Biol Chem, 1998. **273**(7): p. 4135-42.
97. Mori, K., et al., *Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury.* J Clin Invest, 2005. **115**(3): p. 610-21.
98. Parikh, C.R., et al., *Urine IL-18 Is an Early Diagnostic Marker for Acute Kidney Injury and Predicts Mortality in the Intensive Care Unit.* Journal of the American Society of Nephrology, 2005. **16**(10): p. 3046-3052.
99. Kinsey, G.R., L. Li, and M.D. Okusa, *Inflammation in acute kidney injury.* Nephron Exp Nephrol, 2008. **109**(4): p. e102-7.
100. Anderson, N.L. and N.G. Anderson, *The Human Plasma Proteome: History, Character, and Diagnostic Prospects.* Molecular Cellular Proteomics, 2002. **1**(11): p. 845-867.
101. Kuk, C., et al., *Mining the ovarian cancer ascites proteome for potential ovarian cancer biomarkers.* Mol Cell Proteomics, 2009. **8**(4): p. 661-9.
102. Gortzak-Uzan, L., et al., *A proteome resource of ovarian cancer ascites: integrated proteomic and bioinformatic analyses to identify putative biomarkers.* J Proteome Res, 2008. **7**(1): p. 339-51.
103. Hewitt, S.M., J. Dear, and R.A. Star, *Discovery of Protein Biomarkers for Renal Diseases.* Journal of the American Society of Nephrology, 2004. **15**(7): p. 1677-1689.
104. Thongboonkerd, V. and P. Malasit, *Renal and urinary proteomics: current applications and challenges.* Proteomics, 2005. **5**(4): p. 1033-42.
105. Olesen, J. and M. Leonardi, *The burden of brain diseases in Europe.* Eur J Neurol, 2003. **10**(5): p. 471-7.
106. Brookmeyer, R., et al., *Forecasting the global burden of Alzheimer's disease.* Alzheimers Dement, 2007. **3**(3): p. 186-91.
107. Hampel, H., et al., *Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives.* Nat Rev Drug Discov, 2010. **9**(7): p. 560-74.
108. Paweletz, C.P., et al., *Application of an end-to-end biomarker discovery platform to identify target engagement markers in cerebrospinal fluid by high resolution differential mass spectrometry.* J Proteome Res, 2010. **9**(3): p. 1392-401.

109. Goonetilleke, U.R., et al., *Proteomic analysis of cerebrospinal fluid in pneumococcal meningitis reveals potential biomarkers associated with survival.* J Infect Dis, 2010. **202**(4): p. 542-50.
110. Zhou, H., et al., *Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery.* Kidney Int, 2006. **69**(8): p. 1471-1476.
111. Knepper, M., et al. *Uroprot*. 2011 [cited 2011 14th January 2011]; Available from: http://www3.niddk.nih.gov/intramural/uroprot/.
112. Havanapan, P.O. and V. Thongboonkerd, *Are protease inhibitors required for gel-based proteomics of kidney and urine?* J Proteome Res, 2009. **8**(6): p. 3109-17.
113. Cheung, A.T., et al., *Safety of lumbar drains in thoracic aortic operations performed with extracorporeal circulation.* The Annals of Thoracic Surgery, 2003. **76**(4): p. 1190-1197.
114. Fedorow, C.A., et al., *Lumbar cerebrospinal fluid drainage for thoracoabdominal aortic surgery: rationale and practical considerations for management.* Anesth Analg, 2010. **111**(1): p. 46-58.
115. Neuhoff, V., et al., *Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250.* Electrophoresis, 1988. **9**(6): p. 255-62.
116. Thery, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.
117. Meng, F., et al., *Quantitative analysis of complex peptide mixtures using FTMS and differential mass spectrometry.* J Am Soc Mass Spectrom, 2007. **18**(2): p. 226-33.
118. Gaeggeler, H.P., et al., *Mineralocorticoid versus glucocorticoid receptor occupancy mediating aldosterone-stimulated sodium transport in a novel renal cell line.* J Am Soc Nephrol, 2005. **16**(4): p. 878-91.
119. *MASCOT Search*. [cited 2010 02/12/2010]; Available from: http://www.matrixscience.com/search_form_select.html.
120. *Harvester*. [cited 2011 24-01-11]; Available from: http://harvester.fzk.de/harvester/.
121. *Online Mendelian Inheritance in Man*. [cited 2011 24-01-11]; Available from: http://www.ncbi.nlm.nih.gov/omim.
122. Mathivanan, S. and R.J. Simpson, *ExoCarta: A compendium of exosomal proteins and RNA.* Proteomics, 2009. **9**(21): p. 4997-5000.
123. *Scipy*. [cited 2010 02/12/2010]; Available from: http://scipy.org/.
124. Hunter, J.D., *Matplotlib: A 2D Graphics Environment.* Computing in Science & Engineering, 2007. **9**(3): p. 90-95.

125. Fliser, D., et al., *Advances in Urinary Proteome Analysis and Biomarker Discovery.* Journal of the American Society of Nephrology, 2007. **18**(4): p. 1057-1071.
126. Thongboonkerd, V., *Practical points in urinary proteomics.* J Proteome Res, 2007. **6**(10): p. 3881-90.
127. Gonzales, P., T. Pisitkun, and M.A. Knepper, *Urinary exosomes: is there a future?* Nephrol Dial Transplant, 2008. **23**(6): p. 1799-801.
128. Perkins, D.N., et al., *Probability-based protein identification by searching sequence databases using mass spectrometry data.* Electrophoresis, 1999. **20**(18): p. 3551-67.
129. Kesimer, M., et al., *Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense.* Faseb J, 2009. **23**(6): p. 1858-68.
130. Mathivanan, S., et al., *Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line LIM1215 reveals a tissue-specific protein signature.* Mol Cell Proteomics, 2010. **9**(2): p. 197-208.
131. Gonzales, P.A., et al., *Large-scale proteomics and phosphoproteomics of urinary exosomes.* J Am Soc Nephrol, 2009. **20**(2): p. 363-79.
132. Choi, D.S., et al., *Proteomic analysis of microvesicles derived from human colorectal cancer cells.* J Proteome Res, 2007. **6**(12): p. 4646-55.
133. Mears, R., et al., *Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry.* Proteomics, 2004. **4**(12): p. 4019-31.
134. Gonzalez-Begne, M., et al., *Proteomic analysis of human parotid gland exosomes by multidimensional protein identification technology (MudPIT).* J Proteome Res, 2009. **8**(3): p. 1304-14.
135. Admyre, C., et al., *Exosomes with immune modulatory features are present in human breast milk.* J Immunol, 2007. **179**(3): p. 1969-78.
136. Wubbolts, R., et al., *Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation.* J Biol Chem, 2003. **278**(13): p. 10963-72.
137. Staubach, S., H. Razawi, and F.G. Hanisch, *Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7.* Proteomics, 2009. **9**(10): p. 2820-35.
138. Looze, C., et al., *Proteomic profiling of human plasma exosomes identifies PPARgamma as an exosome-associated protein.* Biochem Biophys Res Commun, 2009. **378**(3): p. 433-8.
139. Bard, M.P., et al., *Proteomic analysis of exosomes isolated from human malignant pleural effusions.* Am J Respir Cell Mol Biol, 2004. **31**(1): p. 114-21.

140. Hegmans, J.P.J.J., et al., *Proteomic Analysis of Exosomes Secreted by Human Mesothelioma Cells.* American Journal of Pathology, 2004. **164**(5): p. 1807-1815.
141. Koga, K., et al., *Purification, characterization and biological significance of tumor-derived exosomes.* Anticancer Res, 2005. **25**(6A): p. 3703-7.
142. Hart, T.C., et al., *Mutations of the UMOD gene are responsible for medullary cystic kidney disease 2 and familial juvenile hyperuricaemic nephropathy.* J Med Genet, 2002. **39**(12): p. 882-92.
143. Schmitz, C., et al., *Megalin deficiency offers protection from renal aminoglycoside accumulation.* J Biol Chem, 2002. **277**(1): p. 618-22.
144. Kantarci, S., et al., *Mutations in LRP2, which encodes the multiligand receptor megalin, cause Donnai-Barrow and facio-oculo-acoustico-renal syndromes.* Nat Genet, 2007. **39**(8): p. 957-9.
145. Groenestege, W.M., et al., *Impaired basolateral sorting of pro-EGF causes isolated recessive renal hypomagnesemia.* J Clin Invest, 2007. **117**(8): p. 2260-7.
146. Debiec, H., et al., *Antenatal membranous glomerulonephritis due to anti-neutral endopeptidase antibodies.* N Engl J Med, 2002. **346**(26): p. 2053-60.
147. Koch, J., et al., *Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase. Identification Of the first molecular lesion causing Farber disease.* J Biol Chem, 1996. **271**(51): p. 33110-5.
148. Bar, J., et al., *Molecular analysis of acid ceramidase deficiency in patients with Farber disease.* Hum Mutat, 2001. **17**(3): p. 199-209.
149. Simon, D.B., et al., *Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter.* Nat Genet, 1996. **12**(1): p. 24-30.
150. Venta, P.J., et al., *Carbonic anhydrase II deficiency syndrome in a Belgian family is caused by a point mutation at an invariant histidine residue (107 His----Tyr): complete structure of the normal human CA II gene.* Am J Hum Genet, 1991. **49**(5): p. 1082-90.
151. Gribouval, O., et al., *Mutations in genes in the renin-angiotensin system are associated with autosomal recessive renal tubular dysgenesis.* Nat Genet, 2005. **37**(9): p. 964-8.
152. Marre, M., et al., *Contribution of genetic polymorphism in the renin-angiotensin system to the development of renal complications in insulin-dependent diabetes: Genetique de la Nephropathie Diabetique (GENEDIAB) study group.* J Clin Invest, 1997. **99**(7): p. 1585-95.
153. Simon, D.B., et al., *Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter NKCC2.* Nat Genet, 1996. **13**(2): p. 183-8.

154. Yoshida, K., et al., *Human beta-galactosidase gene mutations in GM1-gangliosidosis: a common mutation among Japanese adult/chronic cases.* Am J Hum Genet, 1991. **49**(2): p. 435-42.
155. Kretz, K.A., et al., *Characterization of a mutation in a family with saposin B deficiency: a glycosylation site defect.* Proc Natl Acad Sci U S A, 1990. **87**(7): p. 2541-4.
156. Schnabel, D., M. Schroder, and K. Sandhoff, *Mutation in the sphingolipid activator protein 2 in a patient with a variant of Gaucher disease.* FEBS Lett, 1991. **284**(1): p. 57-9.
157. Zhou, H., et al., *Urinary exosomal transcription factors, a new class of biomarkers for renal disease.* Kidney Int, 2008. **74**(5): p. 613-21.
158. Sechi, S. and Y. Oda, *Quantitative proteomics using mass spectrometry.* Current Opinion in Chemical Biology, 2003. **7**(1): p. 70-77.
159. Wang, G., et al., *Label-free protein quantification using LC-coupled ion trap or FT mass spectrometry: Reproducibility, linearity, and application with complex proteomes.* J Proteome Res, 2006. **5**(5): p. 1214-23.
160. Zhou, H., et al., *Exosomal Fetuin-A identified by proteomics: A novel urinary biomarker for detecting acute kidney injury.* Kidney Int, 2006. **70**(10): p. 1847-1857.
161. Kortenoeven, M.L., et al., *Amiloride blocks lithium entry through the sodium channel thereby attenuating the resultant nephrogenic diabetes insipidus.* Kidney Int, 2009. **76**(1): p. 44-53.
162. Blanchard, N., et al., *TCR Activation of Human T Cells Induces the Production of Exosomes Bearing the TCR/CD3/{zeta} Complex.* The Journal of Immunology, 2002. **168**(7): p. 3235-3241.
163. Stoeck, A., et al., *A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44.* Biochem J, 2006. **393**(Pt 3): p. 609-18.
164. Emmanouilidou, E., et al., *Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival.* J Neurosci, 2010. **30**(20): p. 6838-51.
165. Kanno, K., et al., *Urinary Excretion of Aquaporin-2 in Patients with Diabetes Insipidus.* The New England Journal of Medicine, 1995. **332**(23): p. 1540-1545.
166. MacDonald, B.K., et al., *The incidence and lifetime prevalence of neurological disorders in a prospective community-based study in the UK.* Brain, 2000. **123**(4): p. 665-676.
167. Dubois, B., G. Picard, and M. Sarazin, *Early detection of Alzheimer's disease: new diagnostic criteria.* Dialogues Clin Neurosci, 2009. **11**(2): p. 135-9.

168. Pan, S., et al., *A combined dataset of human cerebrospinal fluid proteins identified by multi-dimensional chromatography and tandem mass spectrometry.* Proteomics, 2007. **7**(3): p. 469-73.
169. Boschetti, E. and P.G. Righetti, *The art of observing rare protein species in proteomes with peptide ligand libraries.* Proteomics, 2009. **9**(6): p. 1492-510.
170. Faure, J., et al., *Exosomes are released by cultured cortical neurones.* Mol Cell Neurosci, 2006. **31**(4): p. 642-8.
171. Sharples, R.A., et al., *Inhibition of {gamma}-secretase causes increased secretion of amyloid precursor protein C-terminal fragments in association with exosomes.* The FASEB Journal, 2008. **22**(5): p. 1469-1478.
172. Kokubo, H., et al., *Part of membrane-bound A[beta] exists in rafts within senile plaques in Tg2576 mouse brain.* Neurobiology of Aging, 2005. **26**(4): p. 409-418.
173. Stoorvogel, W., et al., *The Biogenesis and Functions of Exosomes.* Traffic, 2002. **3**(5): p. 321-330.
174. Mangialasche, F., et al., *Alzheimer's disease: clinical trials and drug development.* Lancet Neurol, 2010. **9**(7): p. 702-16.
175. Garcia-Alloza, M., et al., *Existing plaques and neuritic abnormalities in APP:PS1 mice are not affected by administration of the gamma-secretase inhibitor LY-411575.* Mol Neurodegener, 2009. **4**: p. 19.
176. Das, P., et al., *Reduced effectiveness of Abeta1-42 immunization in APP transgenic mice with significant amyloid deposition.* Neurobiol Aging, 2001. **22**(5): p. 721-7.
177. Levites, Y., et al., *Anti-Abeta42- and anti-Abeta40-specific mAbs attenuate amyloid deposition in an Alzheimer disease mouse model.* J Clin Invest, 2006. **116**(1): p. 193-201.
178. Osborn, G.G. and A.V. Saunders, *Current treatments for patients with Alzheimer disease.* J Am Osteopath Assoc, 2010. **110**(9 Suppl 8): p. S16-26.
179. Citron, M., *Alzheimer's disease: strategies for disease modification.* Nat Rev Drug Discov, 2010. **9**(5): p. 387-98.
180. Dubois, B., et al., *Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria.* Lancet Neurol, 2007. **6**(8): p. 734-46.
181. Schrader, W. and H.W. Klein, *Liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR MS): an early overview.* Anal Bioanal Chem, 2004. **379**(7-8): p. 1013-24.
182. Marshall, A.G., C.L. Hendrickson, and G.S. Jackson, *Fourier transform ion cyclotron resonance mass spectrometry: a primer.* Mass Spectrom Rev, 1998. **17**(1): p. 1-35.

183. Manza, L.L., et al., *Sample preparation and digestion for proteomic analyses using spin filters.* Proteomics, 2005. **5**(7): p. 1742-5.
184. Blacker, D., et al., *Alpha-2 macroglobulin is genetically associated with Alzheimer disease.* Nat Genet, 1998. **19**(4): p. 357-60.
185. Fox, J.W., et al., *Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia.* Neuron, 1998. **21**(6): p. 1315-25.
186. Walther, M., et al., *Galectin-3 is upregulated in microglial cells in response to ischemic brain lesions, but not to facial nerve axotomy.* J Neurosci Res, 2000. **61**(4): p. 430-5.
187. Venkatesan, C., et al., *Chronic upregulation of activated microglia immunoreactive for galectin-3/Mac-2 and nerve growth factor following diffuse axonal injury.* J Neuroinflammation, 2010. **7**: p. 32.
188. Lukk, M., et al., *A global map of human gene expression.* Nat Biotechnol, 2010. **28**(4): p. 322-4.
189. Su, A.I., et al., *A gene atlas of the mouse and human protein-encoding transcriptomes.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6062-7.
190. Hornemann, T., et al., *Cloning and initial characterization of a new subunit for mammalian serine-palmitoyltransferase.* J Biol Chem, 2006. **281**(49): p. 37275-81.
191. Kramer-Albers, E.M., et al., *Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons?* Proteomics Clin Appl, 2007. **1**(11): p. 1446-61.
192. Polaskova, V., et al., *High-abundance protein depletion: comparison of methods for human plasma biomarker discovery.* Electrophoresis, 2010. **31**(3): p. 471-82.
193. Kim, K. and Y. Kim, *Preparing multiple-reaction monitoring for quantitative clinical proteomics.* Expert Rev Proteomics, 2009. **6**(3): p. 225-9.
194. Kitteringham, N.R., et al., *Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics.* J Chromatogr B Analyt Technol Biomed Life Sci, 2009. **877**(13): p. 1229-39.
195. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nat Cell Biol, 2007. **9**(6): p. 654-659.
196. Taylor, D.D. and C. Gercel-Taylor, *MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer.* Gynecologic Oncology, 2008. **110**(1): p. 13-21.
197. Bonassi, S., M. Neri, and R. Puntoni, *Validation of biomarkers as early predictors of disease.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2001. **480-481**: p. 349-358.

198. Lock, E.A. and J.V. Bonventre, *Biomarkers in translation; past, present and future.* Toxicology, 2008. **245**(3): p. 163-6.
199. Hoffman, S.A., et al., *Higher dimensional (Hi-D) separation strategies dramatically improve the potential for cancer biomarker detection in serum and plasma.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **849**(1-2): p. 43-52.
200. Tang, J., et al., *Recent development of multi-dimensional chromatography strategies in proteome research.* J Chromatogr B Analyt Technol Biomed Life Sci, 2008. **866**(1-2): p. 123-32.
201. Nagele, E., et al., *2D-LC/MS techniques for the identification of proteins in highly complex mixtures.* Expert Rev Proteomics, 2004. **1**(1): p. 37-46.
202. Gilar, M., et al., *Orthogonality of separation in two-dimensional liquid chromatography.* Anal Chem, 2005. **77**(19): p. 6426-34.
203. Rogers, L.D. and L.J. Foster, *Phosphoproteomics--finally fulfilling the promise?* Mol Biosyst, 2009. **5**(10): p. 1122-9.
204. Grimsrud, P.A., et al., *Phosphoproteomics for the masses.* ACS Chem Biol. **5**(1): p. 105-19.
205. Zhang, L., H. Lu, and P. Yang, *Specific enrichment methods for glycoproteome research.* Anal Bioanal Chem, 2010. **396**(1): p. 199-203.
206. Zeng, X., et al., *Lung cancer serum biomarker discovery using glycoprotein capture and liquid chromatography mass spectrometry.* J Proteome Res, 2010. **9**(12): p. 6440-9.
207. Miranda, K.C., et al., *Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease.* Kidney Int, 2010. **78**(2): p. 191-9.
208. Silverman, J.M., et al., *An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages.* Journal of Cell Science: p. jcs.056465.
209. van der Pol, E., et al., *Optical and non-optical methods for detection and characterization of microparticles and exosomes.* J Thromb Haemost. **8**(12): p. 2596-607.
210. Savina, A., et al., *Exosome Release Is Regulated by a Calcium-dependent Mechanism in K562 Cells.* Journal of Biological Chemistry, 2003. **278**(22): p. 20083-20090.
211. Feng, D., et al., *Cellular Internalization of Exosomes Occurs Through Phagocytosis.* Traffic, 2010.
212. Sheldon, H., et al., *New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes.* Blood. **116**(13): p. 2385-94.
213. Ristorcelli, E., et al., *Human tumor nanoparticles induce apoptosis of pancreatic cancer cells.* The FASEB Journal, 2008: p. fj.07-102855.

214. Hogan, M.C., et al., *Characterization of PKD Protein-Positive Exosome-Like Vesicles.* Journal of the American Society of Nephrology, 2009. **20**(2): p. 278-288.
215. Saunderson, S.C., et al., *Induction of Exosome Release in Primary B Cells Stimulated via CD40 and the IL-4 Receptor.* The Journal of Immunology, 2008. **180**(12): p. 8146-8152.
216. Mathivanan, S., H. Ji, and R.J. Simpson, *Exosomes: extracellular organelles important in intercellular communication.* J Proteomics. **73**(10): p. 1907-20.
217. Schorey, J.S. and S. Bhatnagar, *Exosome Function: From Tumor Immunology to Pathogen Biology.* Traffic. **0**(0): p.???-???
218. Camussi, G., et al., *Exosomes/microvesicles as a mechanism of cell-to-cell communication.* Kidney Int.
219. Lynch, S., et al., *Novel MHC Class I Structures on Exosomes.* The Journal of Immunology, 2009. **183**(3): p. 1884-1891.
220. Montecalvo, A., et al., *Exosomes As a Short-Range Mechanism to Spread Alloantigen between Dendritic Cells during T Cell Allorecognition.* The Journal of Immunology, 2008. **180**(5): p. 3081-3090.
221. Pegtel, D.M., et al., *Functional delivery of viral miRNAs via exosomes.* Proceedings of the National Academy of Sciences.
222. Smalheiser, N.R., *Exosomal transfer of proteins and RNAs at synapses in the nervous system.* Biol Direct, 2007. **2**(1): p. 35.
223. Smalheiser, N.R., *Do Neural Cells Communicate with Endothelial Cells via Secretory Exosomes and Microvesicles?* Cardiovasc Psychiatry Neurol, 2009. **2009**: p. 383086.
224. Baj-Krzyworzeka, M., et al., *Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes.* Cancer Immunology, Immunotherapy, 2006. **55**(7): p. 808-818.
225. Gibbings, D.J., et al., *Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity.* Nat Cell Biol, 2009. **11**(9): p. 1143-9.
226. Lee, Y.S., et al., *Silencing by small RNAs is linked to endosomal trafficking.* Nat Cell Biol, 2009. **11**(9): p. 1150-6.
227. Ohshima, K., et al., *Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line.* PLoS One. **5**(10): p. e13247.
228. Michael, A., et al., *Exosomes from human saliva as a source of microRNA biomarkers.* Oral Dis, 2009.

229. Anderson, N.L., et al., *The Human Plasma Proteome: A Nonredundant List Developed by Combination of Four Separate Sources.* Molecular Cellular Proteomics, 2004. **3**(4): p. 311-326.
230. Bell, A.W., et al., *A HUPO test sample study reveals common problems in mass spectrometry-based proteomics.* Nat Meth, 2009. **advanced online publication**.
231. Saha, S., et al., *HIP2: an online database of human plasma proteins from healthy individuals.* BMC Med Genomics, 2008. **1**: p. 12.
232. Omenn, G.S., et al., *Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database.* Proteomics, 2005. **5**(13): p. 3226-45.
233. Mortensen, P., et al., *MSQuant, an open source platform for mass spectrometry-based quantitative proteomics.* J Proteome Res, 2010. **9**(1): p. 393-403.
234. Gaeggeler, H.P., et al., *Vasopressin-dependent coupling between sodium transport and water flow in a mouse cortical collecting duct cell line.* Kidney Int, 2011. **79**(8): p. 843-52.

Appendix 1 – GeLC-MS/MS protein identification of urinary exosomal proteins

Additional information on any protein identifications made by one ion in any single band is presented. The m/z ratio, charge and sequence of the peptide are included. The presence of any peptides linked to the identified protein in multiple bands or in a combined analysis of all bands is indicated.

Protein	Accession ID	Maximum ions per band	Mascot Score	Presence in multiple bands	m/z	Charge	Peptide
Carbonic anhydrase II	NP_000058	1	93	Only one ion detected in a single band	835.318	2	K.AVQQPDGLAVLGIFLK.V
Tyrosine 3/tryptophan 5 -monooxygenase activation protein, epsilon polypeptide	NP_006752	1	93	Two ions detected in the combined analysis	1044.535	2	K.AAFDDAIAELDTLSEESYK.D
					973.062	2	K.LAEQAERYDEMVESMK.K
Calbindin D28K	AAB19408	1	76	Only one ion detected in a single band	792.243	2	K.ELQNLIQELQQAR.K
Alpha globin	CAA23749	1	55	Only one ion detected in a single band	765.142	2	K.VGAHAGEYGAEALER.H
Phosphoglycerate kinase 2	CAA28872	1	49	Two ions detected in the combined analysis	818.068	2	K.LGDVYVNDAFGTAHR.A
					467.801	2	K.ALMDEIVK.A
Gamma-glutamyltransferase 1	AAA52546	1	122	Four ions detected in combined analysis	701.706	2	R.NIDQAVTAALETR.H
					938.974	2	R.TVIEQQPVLCEVFCR.D
					577.434	2	K.GGLSVAVPGEIR.G
					887.653	1	K.GLAAALENK.R
Bile salt export pump	AAD28285	1	120	Three ions detected in combined analysis	924.53	2	K.ILLLDEATSALDTESEK.T
					672.219	2	K.EIPMERVIAAAK.Q
					994.189	2	K.QALEMVGQITNEALSNIR.T

Orphan G-protein coupled receptor (GPRC5B)	AAF67321	1	66	Ion detected in two bands	717.736	2	R.ETAFEEDVQLPR.A
Napsin A preproprotein	NP_004842	1	80	Only one ion detected in a single band	1088.087	2	K.GCAAILDTGTSLITGPTEEIR.A
Aquaporin 1	AAC16481	1	77	Only one ion detected in a single band	824.119	2	K.YPVGNNQTAVQDNVK.V
Desmoglein-1 precursor	Q02413	1	76	Two ions detected in the combined analysis	818.729	2	K.YQGTILSIDDNLQR.T
					754.931	3	K.VVKPLDYEAMQSLQLSIGVR.N
CD26	CAA43118	1	48	Three ions detected in combined analysis	789.739	2	R.LGTFEVEDQIEAAR.Q
					749.072	2	K.LAYVWNNDIYVK.I
					969.171	2	K.FWYQMILPPHFDKSK.K
Tetraspan TM4SF (Tspan-1)	AAC69714	1	45	Only one ion detected in a single band	814.332	2	K.VEGCFNQLLYDIR.T
Apolipoprotein J precursor	AAA51765	1	60	Only one ion detected in a single band	645.113	2	R.ELDESLQVAER.L
CD59 antigen p18-20	NP_000602	1	50	Only one ion detected in a single band	770.572	2	K.FEHCNFNDVTTR.L
Lysosomal pepstatin insensitive protease	AAB80725	1	101	Two ions detected in combined analysis	992.156	2	R.AYPDVAALSDGYWVVSNR.V
					803.147	2	R.LYQQHGAGLFDVTR.G

Beta-trace 23.5 kda glycoprotein	AAB24507	1	46	Only one ion detected in a single band	893.115	2	-.APEAQVSVQPNFQQDK.F

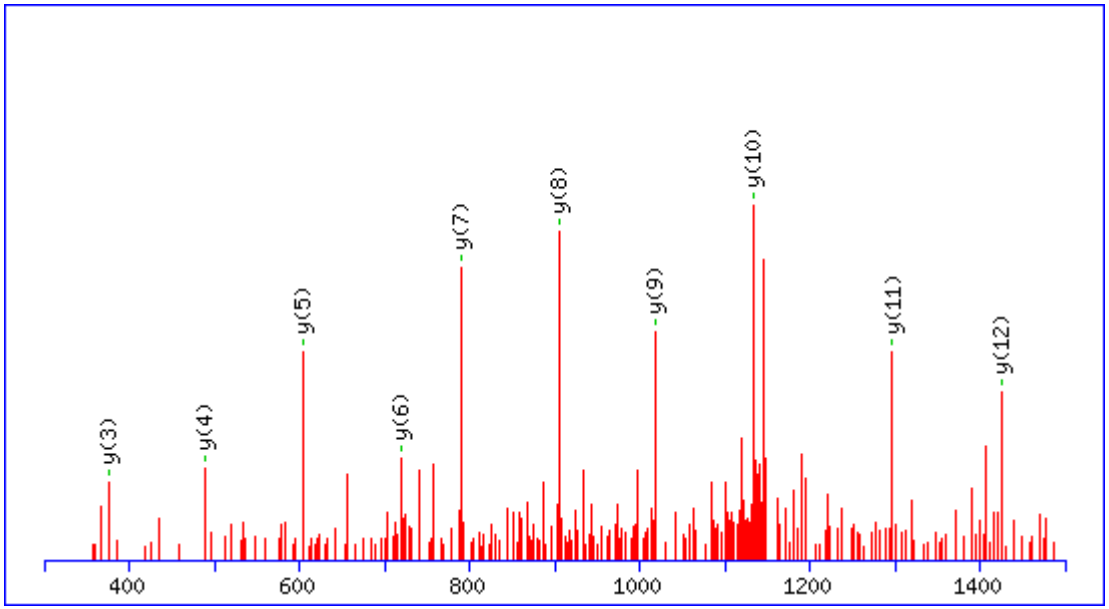
Appendix 2 – LC-MS/MS protein identification of CSF exosomal proteins

Additional information on any protein identifications made by one ion is presented. The m/z ratio, charge and sequence of the peptide are included. For each peptide the fragmentation spectra is shown and the peaks used to identify the peptide indicated.

Accession	Name	Mascot score	Seq coverage (%)	m/z	z	Peptide
P29972	Aquaporin-1	85	7	1156.5566	2	VWTSGQVEEYDLDADDINSR
P00480	Ornithine carbamoyltransferase, mitochondrial	43	2	495.7828	2	QSDLDTLAK
Q9P2M7	Cingulin	43	0	379.3114	2	IEVLQR
Q68D51	DENN domain-containing protein 2C	40	0	359.2776	2	NSQTIR
P02741	C-reactive protein	39	4	564.8655	2	ESDTSYVSLK
Q8TF72	Shroom 3	38	0	466.7784	2	SSPATADKR
P07205	Phosphoglycerate kinase 2	37	2	503.7981	2	DCVGAEVEK
Q92911	Sodium/iodide cotransporter	37	2	800.0805	2	STLAPGLLWWDLAR

P29972

Peptide: VWTSGQVEEYDLDADDINSR

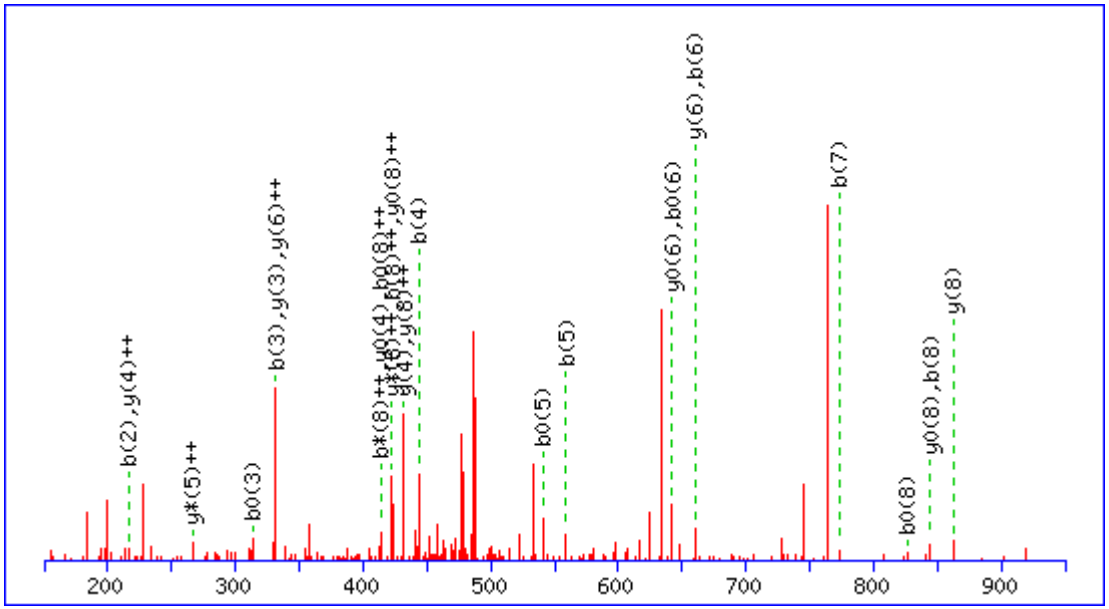


#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	100.0757	50.5415					V							20
2	286.1550	143.5811					W	2212.9629	1106.9851	2195.9364	1098.4718	2194.9523	1097.9798	19
3	387.2027	194.1050			369.1921	185.0997	T	2026.8836	1013.9454	2009.8571	1005.4322	2008.8730	1004.9402	18
4	474.2347	237.6210			456.2241	228.6157	S	1925.8359	963.4216	1908.8094	954.9083	1907.8254	954.4163	17
5	531.2562	266.1317			513.2456	257.1264	G	1838.8039	919.9056	1821.7773	911.3923	1820.7933	910.9003	16
6	659.3148	330.1610	642.2882	321.6477	641.3042	321.1557	Q	1781.7824	891.3949	1764.7559	882.8816	1763.7719	882.3896	15
7	758.3832	379.6952	741.3566	371.1819	740.3726	370.6899	V	1653.7239	827.3656	1636.6973	818.8523	1635.7133	818.3603	14
8	887.4258	444.2165	870.3992	435.7032	869.4152	435.2112	E	1554.6554	777.8314	1537.6289	769.3181	1536.6449	768.8261	13
9	1016.4684	508.7378	999.4418	500.2245	998.4578	499.7325	E	**1425.6128**	713.3101	1408.5863	704.7968	1407.6023	704.3048	12
10	1179.5317	590.2695	1162.5051	581.7562	1161.5211	581.2642	Y	**1296.5703**	648.7888	1279.5437	640.2755	1278.5597	639.7835	11
11	1294.5586	647.7830	1277.5321	639.2697	1276.5481	638.7777	D	**1133.5069**	567.2571	1116.4804	558.7438	1115.4964	558.2518	10
1	1407.642	704.3250	1390.616	695.8117	1389.632	695.3197	L	**1018.480**	509.7436	1001.453	501.2304	1000.469	500.7383	9

2	7		1		1			**0**		4		4		
13	1522.6696	761.8385	1505.6431	753.3252	1504.6591	752.8332	**D**	**905.3959**	453.2016	888.3694	444.6883	887.3853	444.1963	**8**
14	1593.7067	797.3570	1576.6802	788.8437	1575.6962	788.3517	**A**	**790.3690**	395.6881	773.3424	387.1748	772.3584	386.6828	**7**
15	1708.7337	854.8705	1691.7071	846.3572	1690.7231	845.8652	**D**	**719.3319**	360.1696	702.3053	351.6563	701.3213	351.1643	**6**
16	1823.7606	912.3840	1806.7341	903.8707	1805.7501	903.3787	**D**	**604.3049**	302.6561	587.2784	294.1428	586.2943	293.6508	**5**
17	1936.8447	968.9260	1919.8181	960.4127	1918.8341	959.9207	**I**	**489.2780**	245.1426	472.2514	236.6293	471.2674	236.1373	**4**
18	2050.8876	1025.9474	2033.8611	1017.4342	2032.8771	1016.9422	**N**	**376.1939**	188.6006	359.1674	180.0873	358.1833	179.5953	**3**
19	2137.9197	1069.4635	2120.8931	1060.9502	2119.9091	1060.4582	**S**	262.1510	131.5791	245.1244	123.0659	244.1404	122.5738	**2**
20							**R**	175.1190	88.0631	158.0924	79.5498			**1**

P00480

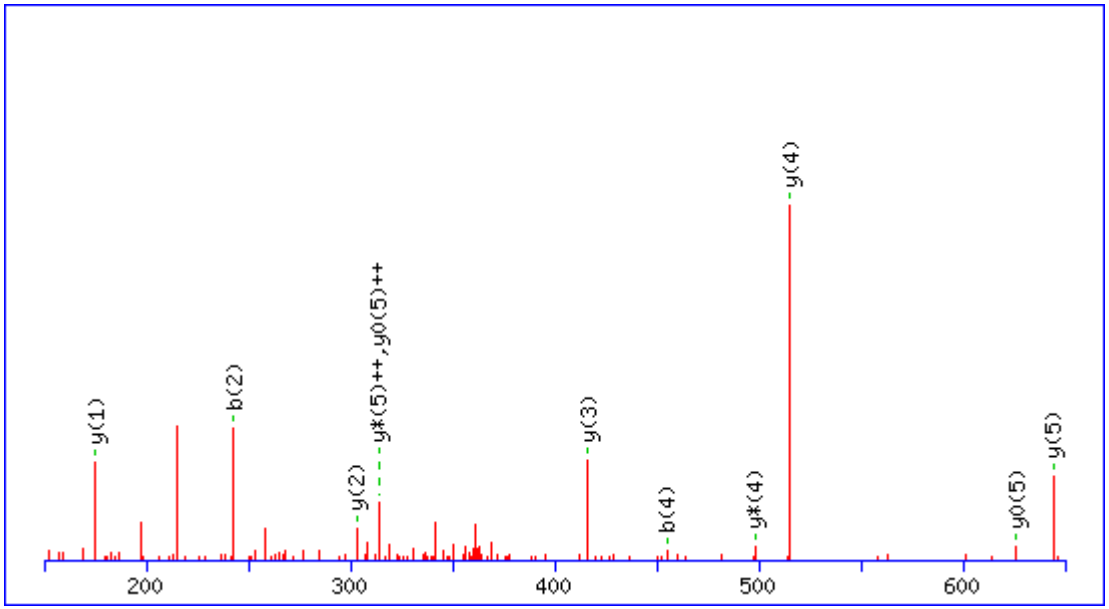
Peptide: QSDLDTLAK



#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	129.0659	65.0366	112.0393	56.5233			Q							9
2	**216.0979**	108.5526	199.0713	100.0393	198.0873	99.5473	S	**862.4516**	**431.7295**	845.4251	**423.2162**	**844.4411**	**422.7242**	8
3	**331.1248**	166.0661	314.0983	157.5528	**313.1143**	157.0608	D	775.4196	388.2134	758.3931	379.7002	757.4090	379.2082	7
4	**444.2089**	222.6081	427.1823	214.0948	426.1983	213.6028	L	**660.3927**	**330.7000**	643.3661	322.1867	**642.3821**	321.6947	6
5	**559.2358**	280.1216	542.2093	271.6083	**541.2253**	271.1163	D	547.3086	274.1579	530.2821	**265.6447**	529.2980	265.1527	5
6	**660.2835**	330.6454	643.2570	322.1321	**642.2729**	321.6401	T	**432.2817**	**216.6445**	415.2551	208.1312	**414.2711**	207.6392	4
7	**773.3676**	387.1874	756.3410	378.6742	755.3570	378.1821	L	**331.2340**	166.1206	314.2074	157.6074			3
8	**844.4047**	**422.7060**	827.3781	**414.1927**	**826.3941**	**413.7007**	A	218.1499	109.5786	201.1234	101.0653			2
9							K	147.1128	74.0600	130.0863	65.5468			1

Q9P2M7

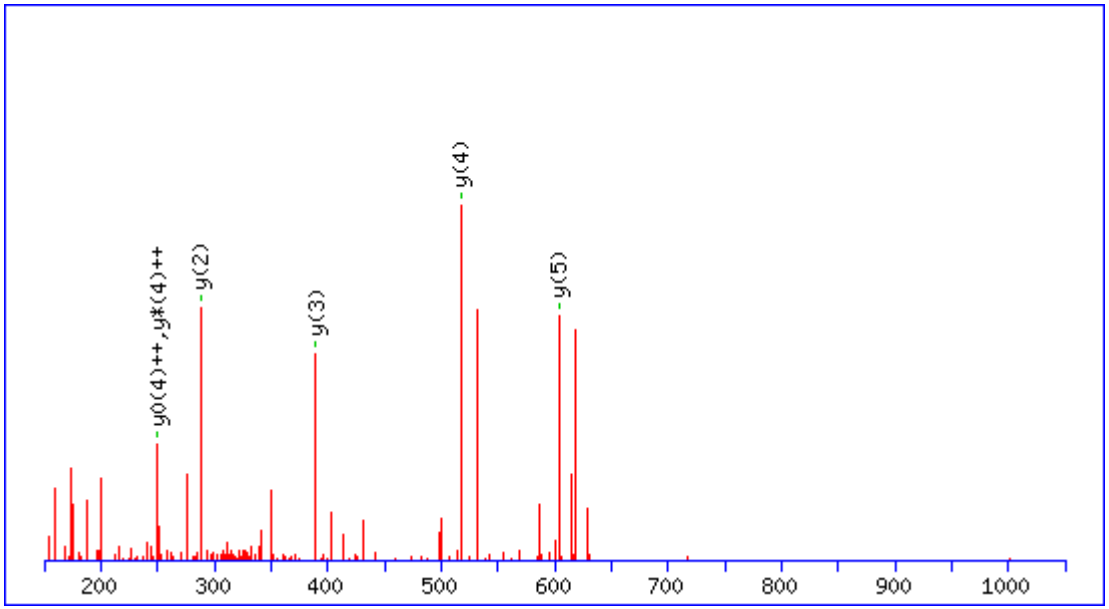
Peptide: IEVLQR



#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	114.0913	57.5493					I							6
2	**243.1339**	122.0706			225.1234	113.0653	E	**644.3726**	322.6899	627.3461	**314.1767**	**626.3620**	**313.6847**	5
3	342.2023	171.6048			324.1918	162.5995	V	**515.3300**	258.1686	**498.3035**	249.6554			4
4	**455.2864**	228.1468			437.2758	219.1416	L	**416.2616**	208.6344	399.2350	200.1212			3
5	583.3450	292.1761	566.3184	283.6629	565.3344	283.1709	Q	**303.1775**	152.0924	286.1510	143.5791			2
6							R	**175.1190**	88.0631	158.0924	79.5498			1

Q68D51

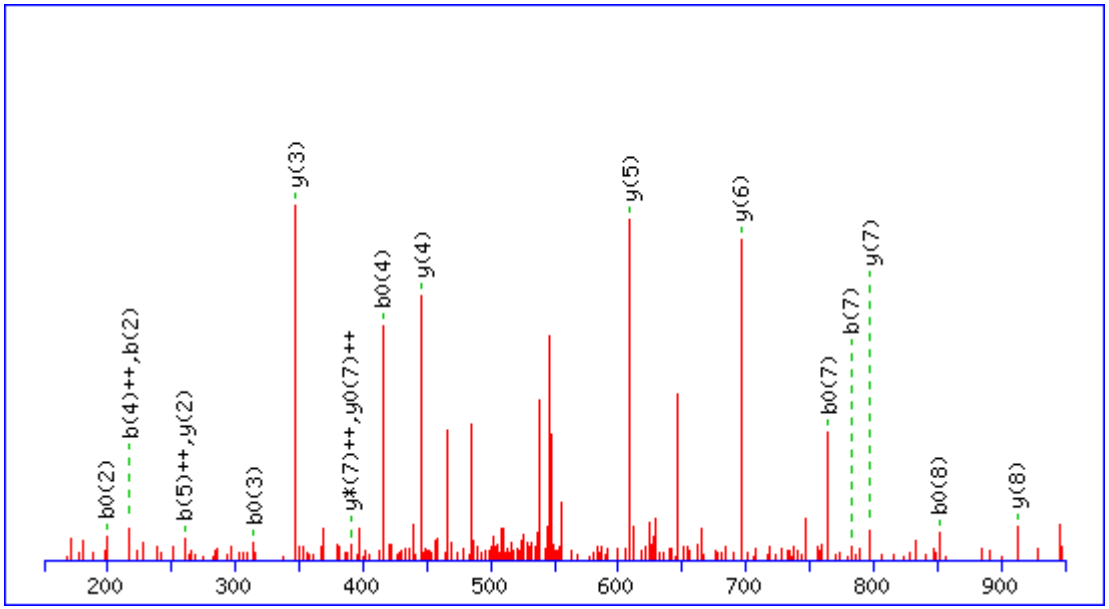
Peptide: NSQTIR



#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	115.0502	58.0287	98.0237	49.5155			N							6
2	202.0822	101.5448	185.0557	93.0315	184.0717	92.5395	S	**604.3413**	302.6743	587.3148	294.1610	586.3307	293.6690	5
3	330.1408	165.5740	313.1143	157.0608	312.1302	156.5688	Q	**517.3093**	259.1583	500.2827	**250.6450**	499.2987	**250.1530**	4
4	431.1885	216.0979	414.1619	207.5846	413.1779	207.0926	T	**389.2507**	195.1290	372.2241	186.6157	371.2401	186.1237	3
5	544.2726	272.6399	527.2460	264.1266	526.2620	263.6346	I	**288.2030**	144.6051	271.1765	136.0919			2
6							R	175.1190	88.0631	158.0924	79.5498			1

P02741

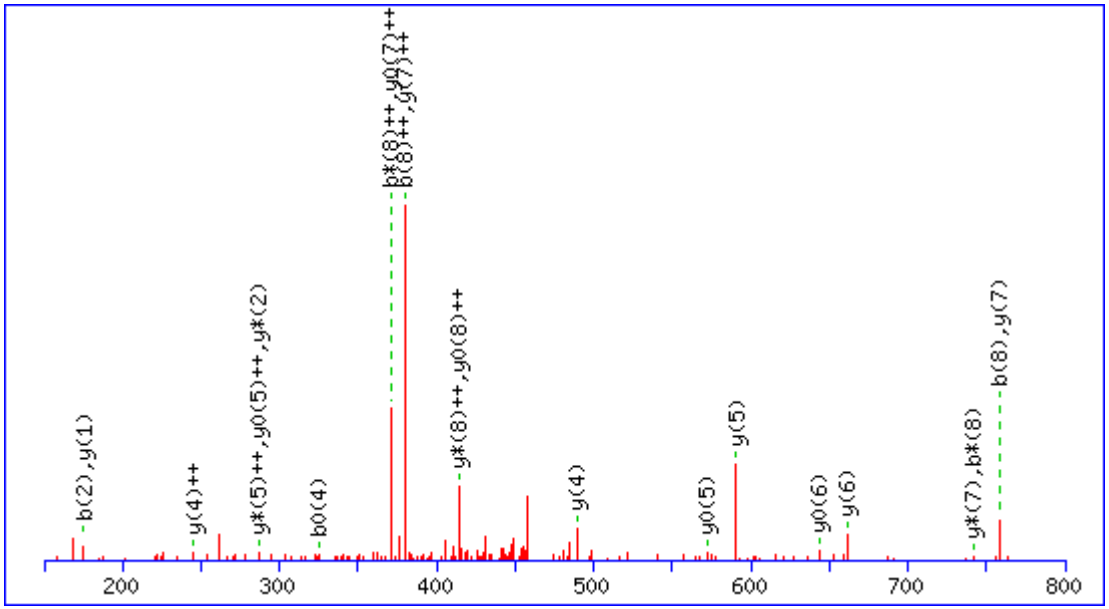
Peptide: ESDTSYVSLK



#	b	b^{++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	130.0499	65.5286	112.0393	56.5233	E							10
2	217.0819	109.0446	199.0713	100.0393	S	999.4993	500.2533	982.4728	491.7400	981.4888	491.2480	9
3	332.1088	166.5581	314.0983	157.5528	D	912.4673	456.7373	895.4407	448.2240	894.4567	447.7320	8
4	433.1565	217.0819	415.1460	208.0766	T	797.4403	399.2238	780.4138	390.7105	779.4298	390.2185	7
5	520.1885	260.5979	502.1780	251.5926	S	696.3927	348.7000	679.3661	340.1867	678.3821	339.6947	6
6	683.2519	342.1296	665.2413	333.1243	Y	609.3606	305.1840	592.3341	296.6707	591.3501	296.1787	5
7	782.3203	391.6638	764.3097	382.6585	V	446.2973	223.6523	429.2708	215.1390	428.2867	214.6470	4
8	869.3523	435.1798	851.3418	426.1745	S	347.2289	174.1181	330.2023	165.6048	329.2183	165.1128	3
9	982.4364	491.7218	964.4258	482.7165	L	260.1969	130.6021	243.1703	122.0888			2
10					K	147.1128	74.0600	130.0863	65.5468			1

Q8TF72

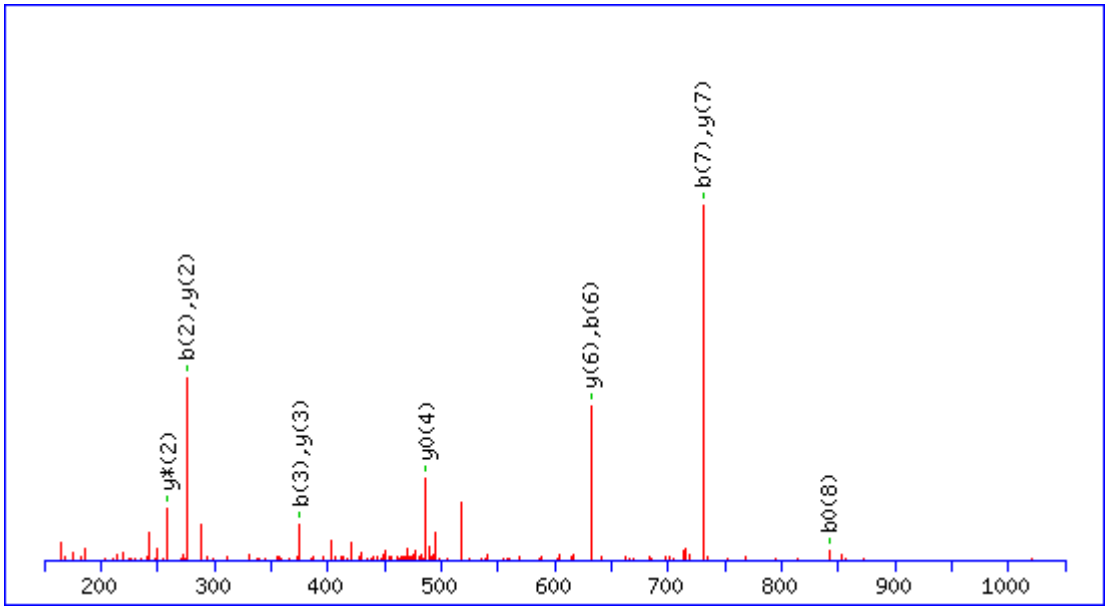
Peptide: SSPATADKR



#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	88.0393	44.5233			70.0287	35.5180	S							9
2	**175.0713**	88.0393			157.0608	79.0340	S	845.4476	423.2274	828.4210	**414.7141**	827.4370	**414.2221**	8
3	272.1241	136.5657			254.1135	127.5604	P	**758.4155**	**379.7114**	**741.3890**	**371.1981**	740.4050	**370.7061**	7
4	343.1612	172.0842			**325.1506**	163.0790	A	**661.3628**	331.1850	644.3362	322.6717	**643.3522**	322.1797	6
5	444.2089	222.6081			426.1983	213.6028	T	**590.3257**	295.6665	573.2991	**287.1532**	**572.3151**	**286.6612**	5
6	515.2460	258.1266			497.2354	249.1214	A	**489.2780**	**245.1426**	472.2514	236.6293	471.2674	236.1373	4
7	630.2729	315.6401			612.2624	306.6348	D	418.2409	209.6241	401.2143	201.1108	400.2303	200.6188	3
8	**758.3679**	**379.6876**	**741.3414**	**371.1743**	740.3573	**370.6823**	K	303.2139	152.1106	**286.1874**	143.5973			2
9							R	**175.1190**	88.0631	158.0924	79.5498			1

P07205

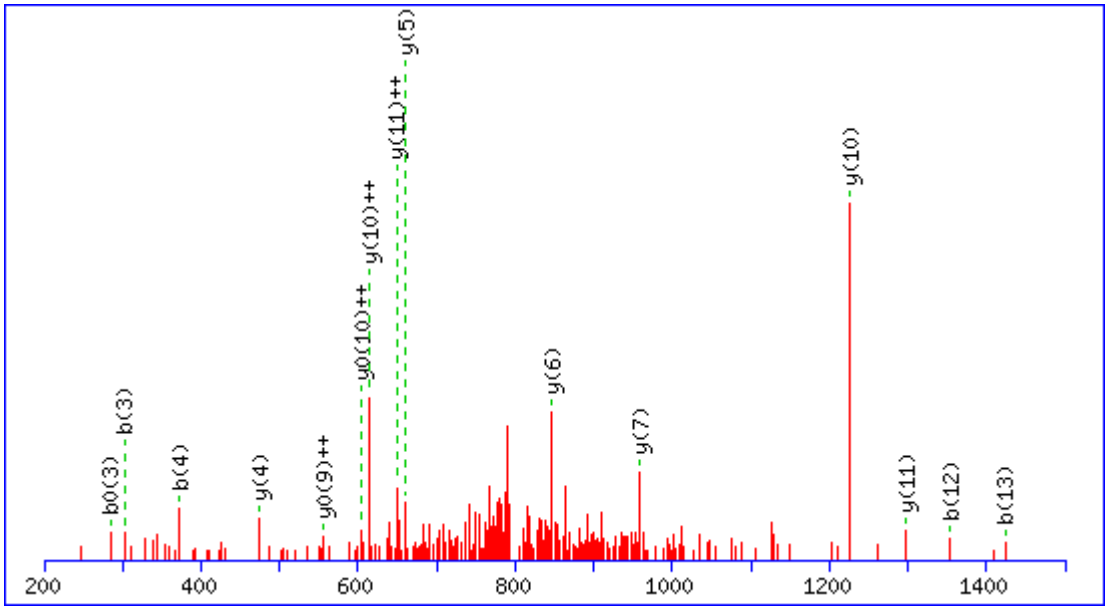
Peptide: DCVGAEVEK



#	b	b^{++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	116.0342	58.5207	98.0237	49.5155	D							9
2	**276.0649**	138.5361	258.0543	129.5308	C	891.4241	446.2157	874.3975	437.7024	873.4135	437.2104	8
3	**375.1333**	188.0703	357.1227	179.0650	V	**731.3934**	366.2003	714.3668	357.6871	713.3828	357.1951	7
4	432.1548	216.5810	414.1442	207.5757	G	**632.3250**	316.6661	615.2984	308.1529	614.3144	307.6608	6
5	503.1919	252.0996	485.1813	243.0943	A	575.3035	288.1554	558.2770	279.6421	557.2930	279.1501	5
6	**632.2345**	316.6209	614.2239	307.6156	E	504.2664	252.6368	487.2399	244.1236	**486.2558**	243.6316	4
7	**731.3029**	366.1551	713.2923	357.1498	V	**375.2238**	188.1155	358.1973	179.6023	357.2132	179.1103	3
8	860.3455	430.6764	**842.3349**	421.6711	E	**276.1554**	138.5813	**259.1288**	130.0681	258.1448	129.5761	2
9					K	147.1128	74.0600	130.0863	65.5468			1

Q92911

Peptide: STLAPGLLWWDLAR



#	b	b^{++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	88.0393	44.5233	70.0287	35.5180	S							14
2	189.0870	95.0471	171.0764	86.0418	T	1511.8369	756.4221	1494.8104	747.9088	1493.8263	747.4168	13
3	**302.1710**	151.5892	**284.1605**	142.5839	L	1410.7892	705.8983	1393.7627	697.3850	1392.7787	696.8930	12
4	**373.2082**	187.1077	355.1976	178.1024	A	**1297.7052**	**649.3562**	1280.6786	640.8429	1279.6946	640.3509	11
5	470.2609	235.6341	452.2504	226.6288	P	**1226.6681**	**613.8377**	1209.6415	605.3244	1208.6575	**604.8324**	10
6	527.2824	264.1448	509.2718	255.1396	G	1129.6153	565.3113	1112.5887	556.7980	1111.6047	**556.3060**	9
7	640.3665	320.6869	622.3559	311.6816	L	1072.5938	536.8006	1055.5673	528.2873	1054.5833	527.7953	8
8	753.4505	377.2289	735.4400	368.2236	L	**959.5098**	480.2585	942.4832	471.7452	941.4992	471.2532	7
9	939.5298	470.2686	921.5193	461.2633	W	**846.4257**	423.7165	829.3991	415.2032	828.4151	414.7112	6
10	1125.6091	563.3082	1107.5986	554.3029	W	**660.3464**	330.6768	643.3198	322.1636	642.3358	321.6715	5
11	1240.6361	620.8217	1222.6255	611.8164	D	**474.2671**	237.6372	457.2405	229.1239	456.2565	228.6319	4
12	**1353.7202**	677.3637	1335.7096	668.3584	L	359.2401	180.1237	342.2136	171.6104			3
13	**1424.7573**	712.8823	1406.7467	703.8770	A	246.1561	123.5817	229.1295	115.0684			2
14					R	175.1190	88.0631	158.0924	79.5498			1

Appendix 3 - Quantitative LC-FT-ICR MS on the CSF exosomal proteome

M/z ratio and charge for each species detected together with the retention time and normalized abundance for the species in each sample is shown in the table.

m/z	Retention time (min)	Retention window (min)	Charge	Normalized abundance					Sample retention time (min)				
				1	2	3	4	5	1	2	3	4	5
659.5042	32.351	0.946	2	93615.42	72355.22	83941.47	89867.90	84749.16	32.351	32.340	32.443	32.406	32.387
615.4762	32.283	1.213	2	71163.90	62386.37	74296.14	77277.90	72466.33	32.283	32.276	32.376	32.346	32.332
637.4901	32.351	1.146	2	84422.49	66849.94	79960.09	82860.75	80988.87	32.351	32.340	32.443	32.406	32.387
593.462	32.283	1.079	2	63779.87	54740.21	65497.96	70420.12	67319.20	32.283	32.276	32.376	32.346	32.332
681.5181	32.351	0.879	2	78253.89	61886.29	74023.22	81999.56	71531.17	32.351	32.340	32.443	32.406	32.387
571.4479	32.216	1.079	2	55120.43	42057.73	54722.79	53133.37	51134.51	32.216	32.212	32.309	32.286	32.278
549.4341	32.216	1.146	2	40206.44	36343.37	42586.65	44967.21	44538.88	32.216	32.212	32.309	32.286	32.278
703.5339	32.418	1.146	2	80279.36	60640.08	69169.68	75640.27	67409.19	32.418	32.404	32.511	32.467	32.441
527.42	32.149	0.946	2	33720.64	23439.49	27400.31	32273.41	29468.34	32.149	32.148	32.241	32.225	32.223
725.5471	32.418	0.744	2	46527.28	38802.53	43252.91	49502.39	47137.49	32.418	32.404	32.511	32.467	32.441
505.4062	32.149	0.674	2	18031.63	15215.96	17339.12	18115.26	17887.61	32.149	32.148	32.241	32.225	32.223
747.5613	32.418	0.475	2	21882.50	19172.58	22106.01	24988.33	21334.74	32.418	32.404	32.511	32.467	32.441
579.4456	29.997	0.531	2	13792.26	11058.62	12324.22	10185.95	10885.83	29.997	30.056	30.095	30.061	30.059
483.3922	32.081	0.538	2	8320.90	6827.74	7289.89	9466.19	7959.34	32.081	32.084	32.174	32.127	32.158
557.4315	29.865	0.464	2	11195.72	7505.80	10420.83	10904.17	9422.82	29.865	29.934	29.960	29.931	29.928
623.4737	30.132	0.540	2	14994.75	13420.90	12840.42	13244.72	14297.62	30.132	30.177	30.230	30.192	30.190
601.4598	30.065	0.538	2	12383.94	12635.61	14659.69	13541.22	14250.50	30.065	30.116	30.162	30.127	30.124
645.4879	30.197	0.338	2	14915.40	13813.61	16110.67	10648.83	12787.44	30.197	30.242	30.297	30.257	30.255
535.4177	29.799	0.464	2	9350.02	7021.29	7744.54	7796.76	7653.47	29.799	29.865	29.893	29.865	29.863
667.5018	30.264	0.405	2	16288.18	12110.31	13133.84	12879.31	11899.28	30.264	30.320	30.364	30.323	30.321
332.802	33.228	0.134	2	6997.25	632.70	0.00	122.29	163.77	33.228	33.233	33.314	33.298	33.260
325.7939	32.960	0.134	2	5999.44	267.49	138.03	129.53	0.00	32.960	32.960	33.046	33.018	32.976
769.5756	32.418	0.338	2	8658.53	9534.59	9511.62	9570.94	11479.14	32.418	32.404	32.511	32.467	32.441
319.316	33.896	0.267	2	773.24	1338.31	1601.25	1616.30	1435.13	33.896	33.912	33.986	33.958	33.926
689.5156	30.333	0.340	2	12898.35	9541.09	10584.42	8554.79	9821.96	30.333	30.404	30.432	30.388	30.386
513.4035	29.734	0.397	2	7962.66	5050.83	6607.53	5451.01	4834.30	29.734	29.793	29.827	29.800	29.798
333.3321	34.299	0.336	2	864.18	1084.99	1461.25	1289.31	1354.98	34.299	34.320	34.388	34.353	34.321
334.3217	33.830	0.270	2	704.70	969.44	1392.88	1056.89	1310.62	33.830	33.844	33.920	33.866	33.871

546.392	24.406	0.606	2	6948.89	7368.71	5254.05	4818.06	4666.68	24.406	24.426	24.465	24.489	24.440
502.3643	24.139	0.467	2	5280.04	5566.87	5977.22	5481.34	4659.59	24.139	24.153	24.199	24.229	24.211
524.3781	24.272	0.467	2	5725.37	7694.27	6372.62	6105.15	6500.01	24.272	24.290	24.330	24.359	24.325
491.3899	29.601	0.333	2	4420.51	2395.54	2664.66	2782.95	3149.76	29.601	29.653	29.694	29.669	29.667
628.9766	32.351	0.337	2	3719.39	4906.42	3754.67	4081.11	3632.90	32.351	32.340	32.443	32.406	32.387
562.9341	32.216	0.404	2	2798.26	3315.05	2844.20	3881.47	3141.42	32.216	32.212	32.309	32.286	32.278
606.9628	32.283	0.337	2	2143.03	3898.61	3828.99	3976.05	2586.26	32.283	32.276	32.376	32.346	32.332
650.9906	32.351	0.337	2	2367.25	3805.18	3380.55	2036.20	4303.49	32.351	32.340	32.443	32.406	32.387
683.9962	32.351	0.202	2	1950.06	1992.84	2207.70	3286.25	2451.66	32.351	32.340	32.443	32.406	32.387
568.4063	24.541	0.337	2	2893.92	5412.71	4761.11	3501.36	3923.36	24.541	24.562	24.600	24.644	24.596
348.3378	34.165	0.269	2	433.72	866.71	817.72	870.57	842.59	34.165	34.183	34.255	34.233	34.189
584.9484	32.283	0.337	2	4595.11	3045.70	4947.84	2785.94	3548.75	32.283	32.276	32.376	32.346	32.332
802.3012	34.030	0.403	3	30840.18	14465.07	13798.31	12499.74	13415.32	34.030	34.049	34.121	34.114	34.057
461.3787	32.081	0.336	2	1867.00	1298.47	2096.96	1808.73	2132.38	32.081	32.084	32.174	32.127	32.158
617.9538	32.283	0.270	2	1941.06	1716.23	2161.54	3683.47	2703.12	32.283	32.276	32.376	32.346	32.332
595.9394	32.283	0.202	2	1396.48	2045.06	1787.94	2349.88	1899.37	32.283	32.276	32.376	32.346	32.332
791.589	32.485	0.271	2	6803.52	8065.43	6928.79	5537.54	3565.10	32.485	32.473	32.578	32.529	32.496
711.53	30.403	0.273	2	5950.06	5350.98	6091.66	6889.48	4079.06	30.403	30.486	30.499	30.454	30.452
673.0046	32.418	0.269	2	3366.25	3513.59	3584.72	5233.62	3564.09	32.418	32.404	32.511	32.467	32.441
540.9207	32.216	0.269	2	1472.00	1437.71	1878.83	2083.41	1507.63	32.216	32.212	32.309	32.286	32.278
573.9257	32.216	0.270	2	1641.89	1448.24	1082.31	1955.68	1360.43	32.216	32.212	32.309	32.286	32.278
1222.552	17.498	0.269	8	0.00	43816.56	0.00	9284.97	3283.08	17.498	17.494	17.553	17.605	17.594
1245.145	25.210	0.202	2	18303.47	8658.81	5893.86	4190.48	0.00	25.210	25.243	25.269	25.349	25.308
661.4893	33.830	0.270	14	1045.59	3484.32	5481.59	3542.37	3073.13	33.830	33.844	33.920	33.866	33.871
1452.524	25.277	0.136	8	3763.52	2564.84	11041.72	0.00	5634.12	25.277	25.310	25.337	25.414	25.376
401.7857	33.497	0.333	2	198.89	1595.75	1009.97	1238.45	749.19	33.497	33.504	33.585	33.576	33.598
698.582	34.030	0.269	13	2330.24	1049.39	1712.98	2067.70	1848.79	34.030	34.049	34.121	34.114	34.057
1412.815	25.210	0.133	6	34473.26	16262.18	15176.44	19860.74	28210.71	25.210	25.243	25.269	25.349	25.308
802.3015	20.518	0.269	5	14511.33	6689.27	9713.37	10324.14	9309.27	20.518	20.575	20.573	20.610	20.636
469.3444	34.367	0.270	10	634.29	1889.90	2119.48	2086.46	2517.79	34.367	34.390	34.455	34.413	34.387
518.9065	32.216	0.202	2	1503.29	1863.28	438.30	571.46	1006.21	32.216	32.212	32.309	32.286	32.278
480.3503	23.872	0.332	2	2381.53	3139.38	2700.10	1982.86	2078.93	23.872	23.885	23.928	23.968	23.969

586.4662	33.562	0.266	11	2970.62	2256.68	3415.49	2855.77	2924.99	33.562	33.571	33.650	33.636	33.653
639.9685	32.351	0.270	2	2287.15	2376.46	1252.72	2886.08	2031.58	32.351	32.340	32.443	32.406	32.387
590.42	24.743	0.269	2	1302.17	3498.68	1694.33	1598.08	2515.38	24.743	24.766	24.800	24.879	24.832
813.6044	32.485	0.271	2	4298.73	3545.89	3613.33	2658.92	4071.78	32.485	32.473	32.578	32.529	32.496
546.4544	32.553	0.205	18	801.63	1638.93	1649.51	656.59	359.09	32.553	32.542	32.645	32.596	32.551
854.6181	34.501	0.271	14	5043.57	5264.73	1938.40	1864.14	5467.83	34.501	34.525	34.588	34.532	34.519
1161.954	25.210	0.133	4	1059.33	1717.11	4293.10	2337.76	5447.02	25.210	25.243	25.269	25.349	25.308
1064.984	16.896	0.135	5	0.00	2908.05	0.00	4528.85	1451.67	16.896	16.852	16.949	16.997	16.975
706.0097	32.351	0.135	2	549.68	1354.64	1697.71	0.00	2234.10	32.351	32.340	32.443	32.406	32.387
661.9831	32.351	0.135	2	3242.00	2468.48	1578.54	2703.89	3279.64	32.351	32.340	32.443	32.406	32.387
1260.559	16.963	0.135	7	0.00	9434.96	0.00	0.00	0.00	16.963	16.925	17.017	17.064	17.044
552.4133	32.216	0.202	11	0.00	505.95	315.96	0.00	557.69	32.216	32.212	32.309	32.286	32.278
1276.89	25.145	0.133	3	1961.86	6460.42	3490.65	11026.89	6965.48	25.145	25.176	25.202	25.284	25.241
1089.076	25.210	0.133	3	38.14	5415.59	4540.32	682.89	0.84	25.210	25.243	25.269	25.349	25.308
1270.474	21.253	0.134	2	0.00	1246.55	0.00	0.00	9407.74	21.253	21.251	21.310	21.302	21.357
1162.48	21.994	0.134	13	0.00	0.00	0.00	0.00	5650.66	21.994	22.032	22.048	22.046	22.139
1567.426	32.216	0.135	8	0.00	0.00	0.00	6610.38	0.00	32.216	32.212	32.309	32.286	32.278
953.3961	20.720	0.135	7	0.00	3349.66	648.74	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1396	21.859	0.135	2	0.00	6108.72	0.00	35.97	0.00	21.859	21.916	21.913	21.885	21.959
1311.893	25.277	0.136	20	0.00	2457.36	1890.56	0.00	3237.08	25.277	25.310	25.337	25.414	25.376
1420.535	19.379	0.135	6	0.00	3209.06	4022.02	0.00	2043.86	19.379	19.406	19.434	19.495	19.512
1213.392	20.787	0.135	5	0.34	11219.01	1076.95	0.00	0.00	20.787	20.835	20.841	20.862	20.899
469.3761	29.534	0.135	2	877.89	1046.57	602.64	751.63	701.01	29.534	29.583	29.627	29.604	29.601
1093.438	32.149	0.134	4	0.00	0.00	0.00	0.00	4529.95	32.149	32.148	32.241	32.225	32.223
918.6971	21.657	0.134	3	0.00	108.86	0.00	2306.43	0.00	21.657	21.707	21.711	21.679	21.690
1336.212	20.787	0.135	6	991.67	5476.34	0.00	0.00	0.00	20.787	20.835	20.841	20.862	20.899
1015.689	21.724	0.135	9	0.00	0.00	0.00	2699.32	0.00	21.724	21.785	21.778	21.742	21.764
1389.738	20.787	0.135	2	0.00	4862.09	0.00	0.00	0.00	20.787	20.835	20.841	20.862	20.899
1292.439	29.865	0.131	4	0.00	4707.34	0.00	0.00	0.00	29.865	29.934	29.960	29.931	29.928
869.8978	21.657	0.134	3	0.00	789.43	520.53	3437.14	0.00	21.657	21.707	21.711	21.679	21.690
1358.967	21.320	0.135	17	0.00	143.25	0.00	0.00	3030.74	21.320	21.324	21.377	21.364	21.409
1014.563	21.657	0.134	10	0.00	41.81	276.00	3038.28	0.00	21.657	21.707	21.711	21.679	21.690

960.3939	21.859	0.135	8	0.00	1634.34	0.00	272.02	0.00	21.859	21.916	21.913	21.885	21.959
1303.59	19.447	0.135	2	0.00	3110.91	0.00	2129.39	0.00	19.447	19.475	19.499	19.563	19.580
2098.386	23.674	0.133	12	0.00	0.00	0.00	6836.82	3134.27	23.674	23.686	23.725	23.774	23.762
1163.469	21.724	0.135	10	0.00	0.00	0.00	4314.84	3166.52	21.724	21.785	21.778	21.742	21.764
867.2157	21.657	0.134	2	0.00	0.00	0.00	2048.30	0.00	21.657	21.707	21.711	21.679	21.690
962.8974	31.141	0.133	17	0.00	0.00	0.00	1520.01	0.00	31.141	31.188	31.236	31.242	31.245
820.8829	21.657	0.134	3	0.00	0.00	0.00	1844.70	0.00	21.657	21.707	21.711	21.679	21.690
1252.014	25.210	0.133	11	0.00	0.00	2977.73	0.00	3220.08	25.210	25.243	25.269	25.349	25.308
1281.304	20.720	0.135	2	0.00	4682.11	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1004.426	21.724	0.135	3	0.00	366.32	0.00	3795.04	0.00	21.724	21.785	21.778	21.742	21.764
1537.138	28.802	0.131	11	0.00	0.00	0.00	0.00	3765.66	28.802	28.852	28.890	28.881	28.887
1208.768	7.198	0.133	2	0.00	5013.25	0.00	0.00	0.00	7.198	7.069	7.243	7.267	7.270
1566.589	20.720	0.135	2	0.00	8497.95	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
734.0469	30.470	0.202	2	1122.89	1352.85	858.01	0.00	0.00	30.470	30.552	30.566	30.519	30.518
1313.936	21.320	0.135	20	0.00	0.00	0.00	0.00	2759.23	21.320	21.324	21.377	21.364	21.409
797.6879	31.409	0.135	6	0.00	1691.69	0.00	0.00	0.00	31.409	31.444	31.504	31.484	31.505
795.9906	21.724	0.135	4	0.00	0.00	0.00	1473.86	0.00	21.724	21.785	21.778	21.742	21.764
1468.103	31.341	0.133	2	0.00	8749.77	3230.81	0.00	0.00	31.341	31.380	31.436	31.423	31.440
811.4185	22.259	0.135	5	0.00	1357.95	0.00	0.00	0.00	22.259	22.264	22.317	22.368	22.377
1244.863	28.802	0.131	15	0.00	0.00	0.00	626.57	4671.98	28.802	28.852	28.890	28.881	28.887
1297.119	20.720	0.135	19	0.00	3172.08	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1404.932	29.000	0.135	12	0.00	4107.11	0.00	0.00	0.00	29.000	29.039	29.092	29.077	29.078
1116.605	21.320	0.202	18	0.00	1969.83	0.00	0.00	2538.00	21.320	21.324	21.377	21.364	21.409
1113.789	28.205	0.131	10	0.00	3877.65	0.00	0.00	0.00	28.205	28.297	28.281	28.292	28.338
1482.733	32.081	0.134	16	0.00	0.00	0.00	2444.68	3217.17	32.081	32.084	32.174	32.127	32.158
1272.878	21.859	0.135	6	1780.17	3307.26	0.00	0.00	0.00	21.859	21.916	21.913	21.885	21.959
1605.481	21.994	0.134	9	0.00	0.00	0.00	0.00	3736.82	21.994	22.032	22.048	22.046	22.139
1398.653	31.341	0.133	11	0.00	3273.26	1462.20	0.00	0.00	31.341	31.380	31.436	31.423	31.440
837.5022	21.657	0.134	2	0.00	794.41	0.00	1793.52	235.84	21.657	21.707	21.711	21.679	21.690
1425.858	21.724	0.135	9	0.00	0.00	2037.33	5685.49	1345.46	21.724	21.785	21.778	21.742	21.764
1416.292	20.787	0.135	15	0.00	2766.53	1756.61	0.00	0.00	20.787	20.835	20.841	20.862	20.899
1439.95	22.259	0.135	18	0.00	2273.04	0.00	0.00	0.00	22.259	22.264	22.317	22.368	22.377

1181.466	21.320	0.135	8	0.00	1515.54	0.00	0.00	3898.76	21.320	21.324	21.377	21.364	21.409
971.3237	21.657	0.134	18	0.00	429.45	0.00	2261.31	0.00	21.657	21.707	21.711	21.679	21.690
1205.143	21.253	0.134	19	0.00	0.00	0.00	0.00	1736.76	21.253	21.251	21.310	21.302	21.357
1216.083	28.933	0.133	7	0.00	3036.36	0.00	0.00	0.00	28.933	28.977	29.024	29.011	29.013
1412.571	29.865	0.131	12	0.00	3345.95	0.00	0.00	0.00	29.865	29.934	29.960	29.931	29.928
843.2562	21.657	0.134	3	0.00	0.00	0.00	4047.74	795.44	21.657	21.707	21.711	21.679	21.690
783.9411	21.657	0.134	9	0.00	0.00	0.00	1537.09	0.00	21.657	21.707	21.711	21.679	21.690
950.2057	21.724	0.135	16	0.00	0.26	0.00	3102.46	0.00	21.724	21.785	21.778	21.742	21.764
1046.669	22.259	0.135	18	838.82	1664.48	0.00	1063.27	0.00	22.259	22.264	22.317	22.368	22.377
1192.757	20.787	0.135	17	0.00	3207.40	0.00	0.00	1164.10	20.787	20.835	20.841	20.862	20.899
1052.436	20.787	0.135	20	808.35	1964.78	0.00	0.00	0.00	20.787	20.835	20.841	20.862	20.899
996.9377	28.270	0.131	3	0.00	2300.34	0.00	0.00	0.00	28.270	28.358	28.349	28.357	28.399
1384.957	28.933	0.133	12	0.00	4044.39	0.00	804.70	0.00	28.933	28.977	29.024	29.011	29.013
1353.284	30.470	0.135	11	0.00	2891.43	0.00	0.00	0.00	30.470	30.552	30.566	30.519	30.518
1245.936	21.657	0.134	14	0.00	0.00	0.00	2033.72	0.00	21.657	21.707	21.711	21.679	21.690
1473.342	21.253	0.134	19	0.00	0.00	0.00	0.00	3030.04	21.253	21.251	21.310	21.302	21.357
1221.121	20.720	0.135	19	0.00	919.51	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
800.8882	20.787	0.135	12	0.00	1085.61	0.00	0.00	0.00	20.787	20.835	20.841	20.862	20.899
2005.284	20.720	0.135	9	0.00	4706.10	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1714.458	20.720	0.135	10	0.00	3603.94	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1236.973	32.283	0.135	3	0.00	3724.24	0.00	0.00	0.00	32.283	32.276	32.376	32.346	32.332
1513.448	28.270	0.131	10	0.00	3093.99	0.00	0.00	0.00	28.270	28.358	28.349	28.357	28.399
1304.315	31.341	0.133	14	2065.73	1771.28	0.00	0.00	0.00	31.341	31.380	31.436	31.423	31.440
971.2511	29.865	0.131	7	0.00	1121.13	0.00	0.00	0.00	29.865	29.934	29.960	29.931	29.928
997.322	22.259	0.135	3	0.00	1788.54	0.00	0.00	0.00	22.259	22.264	22.317	22.368	22.377
956.3617	20.787	0.135	5	0.00	2684.78	0.00	0.00	0.00	20.787	20.835	20.841	20.862	20.899
1316.245	21.724	0.135	11	0.00	0.00	0.00	3012.66	0.00	21.724	21.785	21.778	21.742	21.764
1375.842	20.720	0.135	17	0.00	2178.76	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1779.183	31.409	0.135	9	0.00	2896.74	0.00	0.00	0.00	31.409	31.444	31.504	31.484	31.505
1438.395	20.720	0.135	9	0.00	3798.72	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1220.232	20.787	0.135	4	18.85	2913.12	0.00	0.00	0.00	20.787	20.835	20.841	20.862	20.899
1305.044	32.216	0.135	13	0.00	2956.20	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278

1552.844	31.341	0.133	11	0.00	3823.35	0.00	3083.03	0.00	31.341	31.380	31.436	31.423	31.440
982.1032	22.259	0.135	3	0.00	4052.23	683.53	0.00	443.16	22.259	22.264	22.317	22.368	22.377
1169.702	20.720	0.135	15	0.00	2865.93	566.25	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1094.67	22.191	0.133	2	2011.54	2356.81	24.74	0.00	0.00	22.191	22.205	22.250	22.287	22.317
1813.41	30.470	0.135	11	0.00	3783.79	0.00	0.00	0.00	30.470	30.552	30.566	30.519	30.518
1288.052	20.787	0.135	11	0.00	3111.42	0.00	0.00	0.00	20.787	20.835	20.841	20.862	20.899
1969.687	31.341	0.133	17	0.00	3916.57	0.00	0.00	0.00	31.341	31.380	31.436	31.423	31.440
1241.583	32.216	0.135	18	0.00	2331.13	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278
1039.69	22.191	0.133	4	375.78	1488.05	0.00	0.00	0.00	22.191	22.205	22.250	22.287	22.317
1154.465	28.205	0.131	15	0.00	1747.61	0.00	0.00	0.00	28.205	28.297	28.281	28.292	28.338
1408.574	32.216	0.135	12	2126.45	2655.85	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278
866.069	21.657	0.134	2	0.00	91.36	0.00	2506.26	0.00	21.657	21.707	21.711	21.679	21.690
1715.638	32.216	0.135	13	0.00	3901.60	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278
1215.061	22.191	0.133	11	0.00	2344.37	0.00	0.00	0.00	22.191	22.205	22.250	22.287	22.317
1244.685	32.216	0.135	2	0.00	1945.92	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278
1137.733	22.259	0.135	11	0.00	2441.72	0.00	0.00	0.00	22.259	22.264	22.317	22.368	22.377
1299.908	21.320	0.135	12	0.00	0.00	0.00	1925.55	4155.67	21.320	21.324	21.377	21.364	21.409
951.8954	21.657	0.134	20	0.00	0.00	0.00	1633.00	0.00	21.657	21.707	21.711	21.679	21.690
1463.775	32.216	0.135	12	0.00	3035.86	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278
1189.711	32.283	0.135	9	0.00	3088.48	620.97	0.00	159.05	32.283	32.276	32.376	32.346	32.332
1302.26	29.865	0.131	17	0.00	1680.23	0.00	0.00	0.00	29.865	29.934	29.960	29.931	29.928
1505.809	20.720	0.135	15	2445.37	4449.98	6692.08	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1212.033	28.205	0.131	20	0.00	1795.79	0.00	0.00	0.00	28.205	28.297	28.281	28.292	28.338
1557.637	28.205	0.131	13	0.00	2809.00	0.00	0.00	0.00	28.205	28.297	28.281	28.292	28.338
1206.437	20.720	0.135	15	0.00	1825.34	0.00	0.00	0.00	20.720	20.777	20.774	20.799	20.833
1123.427	22.259	0.135	3	1337.64	2219.22	0.00	0.00	1127.07	22.259	22.264	22.317	22.368	22.377
1379.446	22.663	0.135	14	0.00	0.00	0.00	0.00	2077.69	22.663	22.671	22.722	22.739	22.734
579.4346	32.216	0.135	7	0.00	366.89	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278
1030.393	29.930	0.133	12	0.00	1355.15	0.00	1123.36	0.00	29.930	29.995	30.028	29.996	29.994
1432.695	32.216	0.135	14	0.00	1542.38	0.00	0.00	0.00	32.216	32.212	32.309	32.286	32.278
1378.455	28.270	0.131	18	0.00	981.97	0.00	0.00	0.00	28.270	28.358	28.349	28.357	28.399